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ROBERT EMERSON MEMORIAL ISSUE

Robert Emerson 1903–1959

Robert Emerson was born on November 4, 1903, in New York City, into a distinguished New England family. (He was a great-grandson of Ralph Waldo Emerson's older brother). Bob was a typical Yankee —lean, spare, long-headed, long-striding. The family strain was so strong that his brothers and sisters, as well as his three sons, could be easily recognized as members of the Emerson family; and his daughter had the same build. The fact that Bob's wife, Claire Garrison, also came from a distinguished Boston family, must have helped to preserve the type.

Robert Emerson's father, Dr. Haven Emerson, was a well known pioneer in public health; for many years, he has headed the New York City Public Health Service. Haven Emerson was a dedicated, strong-willed, hard-working, stern man; however far his children got away from him in life, or even rebelled against his influence, he left his imprint on them. Bob Emerson used to say that to understand him, one should have known his father. In his eighties, his father not only kept up with his professional work, but spent every moment of his leisure working in the garden of his estate on Long Island. Bob Emerson, too, was a dedicated, hard-working man of strong convictions. He, too, after spending 10 hours reading manometers in the darkness of his laboratory, and walking home on foot as he always did, would throw himself into digging, weeding and pruning in his garden, until darkness forced him indoors. Emerson's backyard was probably the most intensely cultivated plot of land, not only in Urbana, but in all Illinois, resembling Japanese gardens in the careful utilization of every square foot of ground. (He greatly admired the diligence and skill of the Japanese in dealing with soil and plants). The Emerson family lived to a large extent from the produce of their backyard, despising store-bought fruit and vegetables.

The only recreation in which Emerson indulged, was ice skating. He was the animator of the University of Illinois skating rink and its figure skating club. He and his wife—an even better figure skater than Bob—made a handsome couple on ice; and his whole family joined in this pastime.

Thrift was one of the virtues instilled in Emerson in his upbringing. He would buy expensive things if they were truly needed and worth it, but he hated unnecessary spending. He would rather house and feed out-of-town visitors in his own hospitable home than to let them pay what he considered outrageous prices in hotels and restaurants.

Bob Emerson admired perfection in human labor; and he sought perfection in his own work, be it experimentation in photosynthesis, writing a paper, instrument building, wood-carving or fruit growing. He had great respect for workers with pride in their handiwork, and deplored the loss of this pride in the American drive for speed and quantity in production, at the expense of attention to detail and quality. True to his New England tradition—reinforced by his experience as a student in Germany—he believed in the unhurried, self-sufficient, respectable and respected European scholarship, as contrasted with the ways of American academic life, with its constant pressure for rapid results and external recognition. He disliked the development of American universities into what he considered department stores of education, offering for sale every educational good for which there was a demand, and granting an assortment of degrees in most trivial, as well as in truly scholarly subjects. In this as in other fields, he made no secret of his opinions, however much they would antagonize some of his colleagues. In University Senate meetings, when many of us felt unhappy about this or that proposal, but hesitated to start an argument, it was Bob Emerson who stood up and said what he thought without mincing his words—and yet with a disarming modesty and a smile that made more friends than his frankness made enemies. He saw little value in new technical gadgets, intended to free people from the need of doing what he considered an honest day's work, He distrusted new cars with lots of chrome and hundreds of gadgets, and much preferred old, simple, solid touring cars, built to survive hundreds of thousands of miles of hard roads. He preferred the sterner New England climate, and the more puritan New England way of life, to the leisurely ways and soft climate of California. He missed there the change of seasons, in particular the cold, snowy winters of his Eastern childhood.

Emerson considered it one of his missions in life to teach the virtues of integrity, hard work, solid craftsmanship and thrift to his children and to his students. Some resented his didactic, and often sharp remarks. They complained bitterly about his "old

fashioned" views and paternalistic methods; but those who were willing to learn, could—and many did—learn from him not only the fine art of precise experimentation, but also devotion to science, respect for true scholarship, and disdain for external success.

Emerson would not use a University 3-cent stamp for a letter not strictly on University business, and expected the same uncompromising integrity from everybody around him. He would argue before the University Senate that professors should not strive for higher salaries, because raises in wages and salaries are bound to defeat themselves by bringing about inflation; and—he argued—professors, knowing this, should give other classes an example of rational behavior. Some of his listeners were angry and some shrugged their shoulders at such quixotic views; but they also brought him much respect and warm friendship.

Emerson was a pacifist, and a democratic socialist, a friend of Norman Thomas. He taught his children not to fight back when attacked in school or on the street. He felt strongly about economic and racial injustice, and was always on the side of the underdog. He believed that World War II was brought about by economic injustices of the Versailles Treaty, and the post-Versailles policies of the Allies. In the autobiographic note he supplied to the 25th anniversary reunion of his Harvard graduating class, he wrote, "I have seen the strife and violence resulting from economic forces in California during the *Grapes of Wrath* years. I felt sure that economic forces were driving us into war, and that resort to war could not be expected to correct economic world injustice." He continued, "When the war came, I was not inclined to work as a scientist in support of the war effort. Early in the war, I became interested in rubber research, because of the importance of rubber to the United States, and also because I felt that our exploitation of Southeast Asia, where rubber and similarly important products were produced, may have played a large part in stimulating Japan to attack us." This feeling and his indignation "over the attacks of Californians on the civil rights of American citizens of Japanese parentage," led Emerson to his most important venture outside academic life. "I spent the war fostering a program of rubber research in the concentration camps to which the Japanese-Americans were banished. Our aim was to develop the desert shrub, guayule, as a source of rubber which could be produced under American living standards, without resort to the exploitation of native labor in Southeast Asia."

That this work was successful, both as a scientific project, and as a means to give content and purpose to the lives of a number of deportees, was a great source of satisfaction to Emerson. He was greatly distressed when, at the end of the war, the attempts of Japanese to continue the production of guayule rubber on a commercial scale, failed—in his belief—because of opposition by vested interests in the rubber industry.

With such strong feelings about economic and racial injustices of the capitalist system, Emerson's attitude towards the Russian experiment at first was one of sympathetic tolerance. He hated violence from whatever side it came, but it took some time, and reports of his personal friends in Eastern Europe to make him realize that the injustice and violence of Communist totalitarianism was much more inhuman than that of its adversaries.

Emerson's work at the Japanese concentration camp in Owen's Valley during World War II was his most ambitious excursion away from academic life; but in a more private way, he kept helping those he considered oppressed or unfairly treated during all his life. His thriftiness notwithstanding, he quietly loaned considerable amounts of money to individuals who, he believed, deserved it for a start in life—and not always was it repaid. In the last years of his life, he devoted much time to the fight against housing discrimination in his own community. He was always ready to help foreign students, particularly those whose race made it difficult for them to find acceptance and adjust themselves to life in an American community. Perhaps, the strong feeling for the weak and helpless had something to do also with his love for children. I did not know him when his own children (or mine) were small, but I've seen the smile that lit his face when he was permitted to fondle the children of his friends or co-workers. Probably the happiest days of his last year were when he first met his grandson.

Robert Emerson's striving for integrity, reliability, and precision deeply influenced his scientific career. He started studying animal physiology, at Harvard, in 1920, with the intention of following his father and becoming a doctor. Under the influence of W. J. V. Osterhout's lectures on plant physiology, his interest turned from animals to plants, and after receiving a master's degree in Zoology in 1925 and spending a summer at the Harvard tropical laboratory in Cuba, he went to Germany with the intention of studying the formation of chlorophyll in plants. He went to Munich to Richard Willstätter, who had received the Nobel prize for his work on chlorophyll and photosynthesis, but found him in conflict with the University because of antisemitic activities of students and faculty, and was advised to go to the Kaiser Wilhelm Institute of Biochemistry in Berlin-Dahlem, where another Nobel prize winner, Otto Warburg, was doing pioneer work on quantitative study of photosynthesis. In Warburg's laboratory Emerson learned, to use his own words, "the techniques which I have continued to use" and which "I have taught to those few students who have been so misguided as to subject themselves to my instruction." After 2 years in Warburg's laboratory, Emerson obtained a Ph. D. in Botany at the University of Berlin. Botany was a subject which he did not study extensively in his undergraduate years; and he wrote, "I have not been able to live down my embarrassment at obtaining a Ph. D. in a subject about which I know almost

nothing." (That was written shortly after he had received the Stephan Hale Prize of the American Society of Plant Physiologists in 1949, and shortly before he became a member of the Botany Class of the National Academy of Sciences!)

Emerson returned to Harvard in 1927 as a National Research Council fellow and began to put his newly-acquired knowledge of manometric techniques to use, first in the study of the effects of artificial variations of the chlorophyll content in the green alga Chlorella (achieved by iron, magnesium, or nitrogen deficiency in the nutrient solution), on their capacity for photosynthesis. It was at that time that he married Claire Garrison, who soon became, and has remained, affectionately known as "Tita" to all his colleagues and friends. In 1930, Emerson joined the Biology Department, newly organized by T. H. Morgan at the California Institute of Technology. He stayed in Pasadena for 7 years, and his three sons—Kenneth, Stephen and David—were born there.

Emerson's work at Cal Tech led to the first of his important contributions to the science of photosynthesis. In collaboration with William Arnold,—then an undergraduate student—he carried out experiments on photosynthesis in flashing light, which have by now become classic. Brown and Escombe in England, and Warburg in Germany, had made earlier experiments on the yield of photosynthesis in alternating light with equal light and dark periods, and showed that very short dark periods can contribute to photosynthesis almost as much as equal periods of illumination. Emerson and Arnold achieved decisive progress by substituting flashing for alternating light. They used intense light flashes from condensor discharges, lasting only a few micro-seconds, and varied the length of the dark periods after each flash. This avoided the complications caused by simultaneous change, in both light and dark period. The experiments of Emerson and Arnold led to two fundamental conclusions: 1) that the maximum amount of oxygen produced by a single practically instantaneous flash is—in normal green cells as contrasted to chlorotic cells or aurea varieties—about 1 molecule oxygen for 2000 molecules of chlorophyll, and 2) that this oxygen production occurred, during the dark period, at an exponentially declining rate, with a decay constant of about 100^{-1} sec. Both results remain of fundamental importance for speculations on the kinetic mechanism of photosynthesis; however, the first one has preserved its validity better than the second one. Despite contradictory results by Tamiya and co-workers in Japan (who found up to 3 times greater oxygen yields per flash), Emerson and Arnold's value of the maximum flash yield still appears correct for practically instantaneous flashes (the duration of Tamiya's flashes was of the order of milliseconds). The second conclusion, on the other hand, has since proved to be oversimplified —the decay of oxygen production occurs by a more complicated than a simple first-order law, suggesting a sequence of reactions of different order, with the first-order reaction observed by Emerson and Arnold being but one of them. This complexity probably accounts also for the possibility of obtaining higher flash yields in experiments with longer flashes.

The generally accepted interpretation of the findings of Emerson and Arnold is that photosynthesis requires for its completion an enzyme which is present in the cell in a concentration much lower than that of chlorophyll. (The ratio may be 1 : 2000, or a small multiple of it, depending upon how many molecules of the primary photochemical product are involved in the liberation of a single molecule of oxygen). A more specific interpretation, first suggested by Gaffron and Wohl, postulates that 2000 (or a simple fraction of 2000) chlorophyll molecules are combined in the chloroplast with a single enzyme molecule in a so-called "photosynthetic unit." Even more specifically, it was suggested that this cooperation is achieved by resonance migration of excitation energy from numerous chlorophyll molecules to a single reaction center. This hypothesis plays an important part in modern discussions of the mechanism of photosynthesis, but as yet its correctness could not be either proved or disproved by direct experimental evidence.

In the 1930's doubts had arisen about the correctness of the maximum quantum yield measurements of photosynthesis by Warburg and Negelein in 1921–22. This classical work—the first application of quantum concepts in biology—led to the conclusion that 4 quanta are needed to produce 1 molecule of oxygen. This seemed plausible because 4 hydrogen atoms must be transferred from water to carbon dioxide to reduce the latter to the carbohydrate level. This pluasibility, and the great authority of Warburg as an experimenter, caused general acceptance of his results, and James Franck tried hard to find a thermochemically plausible mechanism of photosynthesis which could function with 4 quanta. Contrary to Warburg's often expressed opinion, it was not the theoretical difficulties encountered by Franck, but the experimental failure of several observers (above all, of Farrington Daniels and co-workers at the University of Wisconsin) to confirm Warburg's and Negelein's findings, using the same biological material (*Chlorella pyrenoidosa*) that first cast doubt on the validity of Warburg's findings, and the feeling that a thorough re-investigation of the important subject of the maximum efficiency of photosynthesis was needed. Emerson undertook this study, taking for this purpose a leave of absence from the California Institute of Technology and spending three and one-half years, beginning 1937, at the Laboratory of Plant Physiology of the Carnegie Institution of Washington, located on the campus of Stanford University. He enjoyed there the sympathetic hospitality of the late Herman A. Spoehr, then director of the laboratory, and the skillful collaboration of Charleton Lewis, his second important collaborator after Arnold. Emerson's daughter, Ruth, was born during this happy period of his life.

Emerson and Lewis developed much improved manometric techniques; in particular, they first applied to the study of photosynthesis the method (orig-

inated by Warburg) of parallel measurements of gas exchange in 2 manometric vessels containing the same quantity of identical algal suspension, but with a different gas : liquid volume ratio. This procedure permitted them to calculate independently the production (or consumption) of the 2 gases, oxygen and carbon dioxide, involved in photosynthesis, instead of relying on the equality of the 2 gas exchanges, derived from the overall stoichiometry of this process (which is what one is forced to do if one uses the simple "one-vessel" method). After long studies, Emerson and Lewis concluded that Warburg and Negelein's results were significantly affected by failure to recognize a gush of carbon dioxide, expelled by cells in the first few minutes of illumination, before steady photosynthesis has set in. When readings made during this transitional period were omitted, the quantum requirements turned out to be between 8 and 12 quanta per molecule oxygen instead of 4. These results were published in 1938–1941; the quantum yield problem seemed to be solved, and Emerson turned his attention to the action spectra of photosynthesis in algae of different families, containing different assortments of pigments.

He returned to Pasadena in January, 1941, and resumed work there; but in December of this year his work was interrupted by America's entry into the war, and all his attention was transferred to the guayule rubber project. This involved not only growing of the guayule shrubs, but also the production of rubber from its juice, carried out by Emerson at the American Rubber Company laboratories in Los Angeles. In this enterprise, Emerson's closest collaborator was Shimpe Nishimura, who brought to this work a combined experience in gardening and in the study of physics at Cal Tech—both brutally interrupted by internment.

Soon after his return to his research work at Cal Tech after the end of the war, Emerson was approached by Neil Stevens, the late head of the Botany Department of the University of Illinois, with a proposal to organize there a research laboratory on photosynthesis. He took Nishimura with him as his assistant, and also asked the University to appoint a physical chemist with an interest in photosynthesis, so that the project could be properly guided both in its plant-physiological and its physico-chemical aspects. Thus began 12 years of a most harmonious collaboration, which Warburg has described as the "Emerson-Rabinowitch photosynthetic unit."

Emerson's relation to theory was ambivalent. On the one hand, he was always conscious of his own lack of training in theoretical physics and physical chemistry and had inordinate respect for all who could operate in these fields. On the other hand, he was fully aware of the poor quantitative reliability of most of the experimental data in biological literature, including even his own measurements (since he has always been his own severest critic); and he felt that theoretical speculation in biology tends to run away from solid experimental foundations. It was difficult, if not impossible to persuade him that even an inexact measurement must have a certain value—a plausible maximum error—which permits one to use it for theoretical speculations, at least within certain limits. If an experiment was not carried out with the greatest precautions as to the consistency of biological material and the precision of all measurements, it was "n.g."—no good—to him, and that was that. This was a constant source of friendly arguments between the two of us, and even more, between him and James Franck, who has brought over from physics into plant physiology the conviction that every measurement must mean something.

In 1948, after Emerson and Lewis' quantum yield results were widely accepted, and confirmed by various studies in other laboratories, which used less precise methods, but were impressive in their consensus, Warburg published a paper in which he reasserted the correctness of his earlier findings, this time on the basis of two-vessel measurements, not subject to Emerson's original criticisms.

Following my suggestion (which I had occasion to regret later) Emerson arranged for Warburg (whose laboratory at Dahlem was inactive at that time due to war losses) to come to the University of Illinois and to attempt the resolution of the discrepancy by cooperative study. Like so many best-laid plans, it all went wrong. Warburg arrived in the summer of 1949, in the midst of the heaviest thunderstorm I have experienced in my 15 years in Urbana; and this proved to be an augury of his stormy stay in Emerson's laboratory. Warburg had been accustomed to work with highly trained technical assistants, and not with colleagues or even graduate students with independent opinions. He was Warburg and he was right. Emerson, at first modest and helpful in his usual way, and full of respect for his famous teacher and guest, also was a stubborn man, particularly when it came to devising experiments, a matter in which he felt he also had great experience and sound judgment. After several months of fitful trys at collaboration, and an unsuccessful attempt to appeal for a third person's arbitration, Warburg left in anger, without saying goodby.

This interlude was hard on Emerson. He was completely convinced of the correctness of his measurements, but his reputation was at stake and everybody expected from him a new study of the quantum yield problem, and interpretation of Warburg's new results. For 15 subsequent years, Emerson's experimental work in Urbana was devoted mainly to this task. Together with Nishimura, and later with other assistants, he went into a detailed study of the manometric techniques. Several interesting findings were obtained, particularly in the demonstration of the complexity of the transitional phenomena in the first minutes of exposure to light and darkness. These results explained some of the discrepancies between Emerson's and Warburg's results. The aim of finding a complete explanation proved elusive, because Warburg, rather than to investigate thoroughly the conditions under

which high quantum yields could be obtained, began publishing increasingly startling observations, whose relation to his own earlier findings was not always made clear, and which made Emerson's control experiments obsolete faster than they could be performed. Warburg's original quantum requirement of 4 (which had a certain plausibility) was replaced by him by 3 and finally by 2.7—the minimum number of quanta needed to conform to the law of conservation of energy. This conclusion was greeted by Warburg as confirmation of his old belief that (to use his words) "in a perfect world, photosynthesis must be perfect," but it seemed entirely implausible to all of us with some respect for the general tenets of reaction kinetics. Furthermore, the high quantum yield, previously described as obtainable only in short experiments in weak light (and which Franck has therefore attempted to attribute to the involvement of respiration intermediates in photosynthesis), was now said to have been obtained also in hour-long runs in strong light, far above the compensation point of respiration and photosynthesis. The presence of a respiration-compensating background illumination was said to be decisive; then, this was changed to a need for a minute, "catalytic" amount of blue light. A very high carbon dioxide concentration, far above the physiological range was announced to be indispensable for obtaining the high quantum yields (although these were supposed to be evidence of physiological perfection of the plant cell!). Finally, photosynthesis was stated to require only a single quantum of light, the rest of the needed energy being provided by respiration, which, according to Warburg, was enormously accelerated during the illumination. Many of these results were directly contradicted, not only by Emerson's experience, but also by those of other observers; none was confirmed. It was this kaleidoscopic change of claims that convinced Emerson that he should confidently leave the field to the judgment of time, and look for a research subject of his own choosing.

Beginning with the work of his student, Tanada, on the action spectrum of photosynthesis in diatoms published in 1951, Emerson resumed the studies he had begun in California with green and blue-green algae (*Chlorella* and *Chroococcus*). For this work, a large monochromator was used, with optical parts originally lent by the Mount Wilson Observatory, which permitted working with much narrower spectral regions than were used by earlier investigators, particularly those who had employed color filters. Much more precise action spectra of photosynthesis could now be obtained, revealing many important details; and the relative efficiency of the quanta absorbed by the different pigments, contained in various photosynthetic cells, could be established with a far improved reliability. The relatively low efficiency of the carotenoids in green and red cells, contrasted with the high efficiency of fucoxanthol and chlorophyll c in diatoms, were among the findings; but perhaps the most important results, obtained in a recent study with Marcia Brody, concerned the efficiency of the phycobilins in the red alga, Porphyridium. Studies of such algae by Blinks, Haxo and Yocum have led to the paradoxical conclusion that chlorophyll a in these organisms is much less effective as sensitizer of photosynthesis than the red pigment, phycoerythrin. Even more paradoxically, a similar result was obtained (by French, and also by Duysens) also in the study of the action spectrum for the excitation of chlorophyll fluorescence; in other words, chlorophyll a in red algae seemed less effective than phycoerythrin in the excitation of its own fluorescence! At the same time, the apparent parallelism of the action spectra of photosynthesis and chlorophyll fluorescence confirmed the wide-spread conviction that energy absorbed by other pigments has to be transferred to chlorophyll a in order to become active in photosynthesis. Several more or less implausible hypotheses were advanced to explain these perverse findings; but Emerson believed that time for speculation will come when the experimental results will be more reliable and systematic. In fact, his studies with M. Brody led to a shift in the experimental basis of speculation; the low efficiency of chlorophyll a in red algae was found to be characteristic not of this pigment as such, but only of its light absorption above 650 mμ; light of shorter wave lengths, also absorbed by chlorophyll a, proved to be more effective than that that absorbed by phycoerythrin; in the limiting case, both pigments were equally effective. The ratio of their efficiency depended on pre-illumination of the algae with light of different colors. The puzzle was not solved, but shifted into an altogether different field.

Already in his work with Lewis, Emerson had noted that a drop in the quantum yield towards the longer waves occurred also in green algae; only there, the decline began much later—beyond 680 mμ, in a region where the absorption of light by chlorophyll declined rapidly, so that the loss of efficiency was much less obvious. In an attempt to find an explanation of this phenomenon of "red drop", Emerson began to study it systematically. The first exciting thing he found was that no red drop occurred when a sufficiently strong background illumination with light of shorter wave length was provided. The finding is superficially reminiscent of some of Warburg's observations, but entirely unrelated to them in that the background light was found effective only in bringing the quantum yield in the far red up to the "normal" level of 8 to 12 quanta per oxygen molecule, and that a considerable intensity of this background light was needed (rather than only "catalytic amounts").

A study of the action spectrum of the "background light" effect led to the striking conclusion that it seemed to be identical with the absorption spectrum of certain "accessory pigments"—chlorophyll b in green algae, phycobilins in red or blue algae (suggesting an explanation of the earlier beginning of the "red drop" in these organisms), and, probably, chlorophyll c in diatoms. In other words, photosynthesis appeared to require, in addition to red light absorbed only by chlorophyll, also light of shorter wave lengths absorbed

either by chlorophyll itself, or by one of the accessory pigments. The interpretation of this highly unexpected result could be sought either in different photochemical functions of the several cell pigments, which must be combined to achieve photosynthesis, or in the existence of 2 or more different forms of excited chlorophyll a—one of which results from direct absorption in the far-red part of the spectrum, while the other can be obtained either by direct absorption of higher frequency quantas by chlorophyll, or by resonance energy transfer of these quanta from the excited accessory pigments. The first interpretation left puzzling the observed parallelism between the action spectra of photosynthesis and of chlorophyll fluorescence; the second left unexplained the striking difference in the position of the red drop in green and in red algae. Thus, the problem remains open, and calls for more experimentation, with the skill and patience Emerson would have applied to it.

In the midst of these experiments, Bob Emerson met sudden death when the plane, carrying him to a conference at Harvard University, missed the LaGuardia runway and plunged into the East River. As part of his dislike of new gadgets, Emerson distrusted airplanes and always advised me against flying. Only in the last few years, when his favorite train from Indianapolis to New York was discontinued, did he grudgingly choose air transportation for his trips to New York. He was booked for another flight, but the lateness of the ill-fated Electra in leaving Chicago made it possible for him to transfer to it at the last moment, hurrying him to his death.

To me, the death of Robert Emerson means the loss of a warm, steady and reliable friend, whose scientific advice was invaluable for me and my students, and for whose opinions in all fields of human interest I had the greatest respect, even if I did not share some of them. The feelings of his numerous and widely-scattered friends are well expressed in a letter from his Harvard friend, Kenneth Thimann, who wrote: "Bob is not a man whom you can ever forget. In some way Bob was the very symbol of uprightness; he loved the truth just as much as he loved the underdog, and he scorned the untruthful and could not have anything to do either with it or with the man who promulgated it. I can imagine his students feeling that they have to judge their lives by what Bob would have done in the circumstances .... Everyone who has come into contact with Bob must have been inspired by him to some degree; it is impossible not to be, just as it is impossible not to remember with clarity his every gesture, his ready smile—often belying fierce disagreement—his enormous ability for friendship and real tenderness. This is a kind of immortality—at least survival for another lifetime—in the memories and even to some extent in the characters of other people, which it is given to very few men to achieve."—EUGENE RABINOWITCH.

LIGHT INDUCED ABSORPTION CHANGES IN PHOTOSYNTHETIC ORGANISMS, II. A SPLIT-BEAM DIFFERENCE SPECTROPHOTOMETER[1]

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Previously (9 to 12), we described a method to observe relatively short lived absorption changes (i.e., having a minimum life time of some 5 milliseconds), induced by brief flashes of strong light.

The sample was intermittently cross-illuminated by a strong actinic beam and its momentary optical density was observed immediately before and immediately after each flash. With a few exceptions (e.g., the 520 shift in green cells and the infra-red effects in purple sulfur bacteria), the absorption changes occurring are so small that the effect of a single flash cannot be observed directly. This difficulty was overcome by the use of a rotating sector disc arrangement, which yields a steady sequence of flashes. A series of transmission values, before and after the individual flashes, then could be collected and averaged so as to yield significant data, even under severe conditions of signal to noise ratio. The rotating disc was, moreover, arranged in such a way that the photocathode was darkened each time a flash hit the sample. Neither scattered actinic light, nor fluorescence induced by it, interfered with the measurements, and the method, therefore, allowed full freedom to vary intensity and color of the actinic beam regardless of the wave length of the light detecting the absorption changes.

Another obvious advantage is the fact that the two observations to be compared are made with a single beam, so that small intensity fluctuations, settling of algae, etc. have relatively slight influence on the readings.

However, a serious limitation of this arrangement was that only variations of absorbance could be observed, which were either reversible (e.g., fast increase by each flash and slow decay in the dark periods) or additive (e.g., increase during each flash without restoration in dark).

[1] March 23, 1959.

Moreover, in most cases it was impossible to discriminate these two types of effects. We therefore felt the need for a better study of slowly occurring absorption changes, i.e., those on top of which eventually the fast phenomena might occur. Especially in the red spectral area—of most importance to photosynthesis—several incongruences exist among observations of "slow" absorption changes by other workers (1, 3, 9) and with our own earlier data on fast changes.

Adhering to the principle of time separation between actinic and detecting beams, we redesigned our apparatus so that a fraction of the detecting beam is guided around the sample and can be used as a reference. Reference and sample beam can be adjusted for equality before actinic illumination is applied. An inequality caused by absorbance changes induced by subsequently given actinic light can then be measured on an absolute rather than a relative basis.

DESCRIPTION OF THE APPARATUS: A schematic illustration of the modified apparatus is shown in figure 1. The sample, contained in a small reaction vessel (R), is cross-illuminated by a flashing actinic beam (arrows) and a continuous detecting beam (dotted) leaving the grating monochromator (Mo). The monochromatic beam is split with the aid of 2 thick hemispherical lenses, ground and positioned as indicated: LL_1. The components of LL_1 are of the type used as a front lens in a microscope condenser (Bleeker Co., Zeist, Holland). Part of each is ground away in order to yield a flat side at an angle of 120° to the front plane. One of these lenses images the monochromator exit slit on the reaction vessel (beam AB), the other images the slit on a photographic circular density wedge W (beam W). The 2 beams are collected and made parallel by a 2nd pair of hemispherical lenses, LL_2, and pass 2 coaxial discs (D_s, D_f). About one third of each lens is ground away perpendicularly to the front to yield a flat side. A thin copperfoil separates the lenses of each pair (i.e., LL_1 and LL_2) and prevents the 2 beams from interfering with each other. A lens of larger diameter, placed closely behind the discs, finally reunites both beams on the cathode of the photomultiplier (P_m). The 2 beams can be adjusted to equal intensity with the aid of the wedge, which for this purpose is provided with a precision vernier (V).

To reduce interferences of phosphorescence, induced mainly by blue actinic illumination, the reaction vessel and the lenses surrounding it were made of quartz.

This compact arrangement with lenses of high aperture allows for optimal collection of the detecting light, which is severely scattered by samples of biological material. The short pathlength through the sample (5 to 10 mm) may be disadvantageous for work with dilute pigment solutions in vitro, but this aspect is immaterial for biological samples, in which the natural pigmentation of the individual chloroplasts is predetermined and generally dense.

One of the 2 rotating discs (D_s) (ca. 500 rpm), located close behind LL_2), serves to transmit alternately beams W and AB onto the photocathode P_m, so that their respective intensities can be compared via a properly arranged electronic detecting system. Synchronous switching of this system was performed with the aid of a 3rd small disc (D_t), mounted on the same axle as D_s (cf. below). The 2nd rotating disc (D_f, closest to the photocell in figure 1) serves to illuminate the sample with the actinic beam in a phosphoroscope arrangement. It is provided with 2 slits, opposed to 2 projections. The slits serve to transmit the actinic beam (twice per revolution), whereas the projections at the same moments blank out the photocell (in order that it not be affected by stray and fluorescent light induced by the flash). Disc D_f spins 4 times faster (ca. 2000 rpm) than the slow disc (D_s), thus yielding a much improved flash shape and—compared to our earlier single disc arrangement—considerably reduces the time-lapse between the flash and the reillumination of the photocathode by the detecting beam. The actinic light source is focussed on a fixed slit, close to D_f, with the aid of a condensing lens. A train of lenses and a mirror concentrate its light on a ca. 5 × 5 mm area of the sample cuvette. A shutter (s) and holders for color or wire gauze filters (FF) are provided in the light path.

The only limitation in the choice of the actinic light source is the hazard of phosphorescence induced, mainly by short wave length light, even in quartz. Interference by these effects, as well as of algal chemiluminiscence, in most cases can be avoided by using strong enough detecting light or, if necessary, cor-

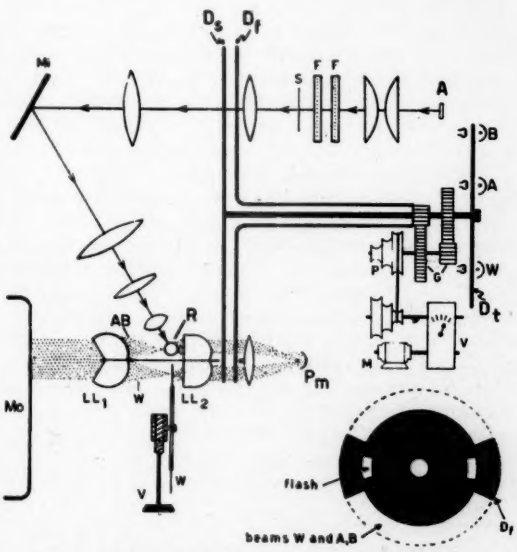


FIG. 1. Schematic illustration of the split-beam apparatus. See text for details. For clarity, baffles, slits, etc., arranged to prevent actinic illumination from reaching the photocell, are not shown. To the lower right the fast disc D_f is shown.

rected for (10). For photosynthesis experiments, incandescent lamps or Xenon arcs are best suited.

A photomultiplier is used as the light detector, since fast time response (10^{-8} sec rise time) and, in several instances, also high sensitivity are required. The signal, generated by the photocell, consists of a series of square pulses—2 per turn of the fast disc, i.e., 8 per turn of the slow disc (cf. fig 3). It is fed into a D.C. amplifier (cf. figure 2), where it is clipped so that only the top part of the pulses—which contain the desired information—appear at the output. The output is fed into 3 identical channels, which can be opened and closed by electronic switches. These switches are operated via photocells actuated by the 3rd rotating disc (D_t, cf. figures 1 and 3). As long as a cut-out in disc (D_t) is in front of a photocell, the corresponding channel is open and the momentary value of the photo-signal is transmitted and stored via an integrating network. For reasons mentioned earlier (11), the primary photo-signal should be kept constant (V_1 in fig 2) in each series of observations. For this purpose the output of one of the channels is amplified and regulates the photomultiplier voltage (V_2, fig 2). In addition this arrangement automatically compensates for slow drifts of the amplifier and of the detecting beam.

Arrangements with Short or Long Dark Times: Two types of measurement have so far been performed with the described set up:

Short dark times: For this type of experiment a slow disc, shaped as shown in figure 3 (D_s top left), was used. Beams AB and W were alternated during equal time periods (8 × per revolution). Synchronously, disc D_t switched from 1 amplifier channel to the other (only 2 channels are needed in this case). Exactly at the moment that disc D_s switched from 1 beam to the other, a separate hole in it coincided with a cut-out in the fast disc—so that a flash hit the sample (and the photocell was briefly darkened). This course of events is illustrated in figure 3 (top right), in which, for clarity of illustration, a large inequality between the signals AB and W is drawn. The time distance between the flashes is about 12 milliseconds, presumably too short to allow the dark reactions to remove much of the photoproducts. These, therefore, will pile up during a sequence of flashes and attain a steady state concentration, close to the level prevailing in strong continuous light. Changes of absorption, eventually induced by such a virtually continuous illumination, now can be detected, if before adding the flashing light, beams AB and W are equalized (with the aid of the wedge W, fig 1), so that the difference recorder yields zero deflection.

Long dark periods: By exchanging the discs D_s and D_t, discussed above for the ones shown in the bottom part of figure 3, a different type of measurement, viz. with flashing light with "long" dark periods, as used in our earlier work, could be performed. Since disc D_s in this case is provided with only 2 holes for transmitting the actinic beam, a flash occurs only 1 out of 4 times a slit in the fast disc passes by. At 500 rpm of D_s, such a coincidence occurs only once per 60 milliseconds. This gives a dark time long enough to allow a considerable decay of short lived photoproducts, even under conditions of steady state flashing light.

The transmission of the sample is observed immediately before the flash (observation B) and again immediately after it (observation A). The desired pattern of observation times is selected by disc D_t and illustrated by the dotted areas in figure 3, bottom right.

During each of the long dark periods there is ample time for making a 3rd observation; namely, of the reference beam W, which is transmitted by disc D_s during half the cycle (one quarter revolution). The trigger disc is provided with only 1 cut-out per cycle (equal to one half revolution), which at the proper moments opens the amplifier channels denoted B, A and W.

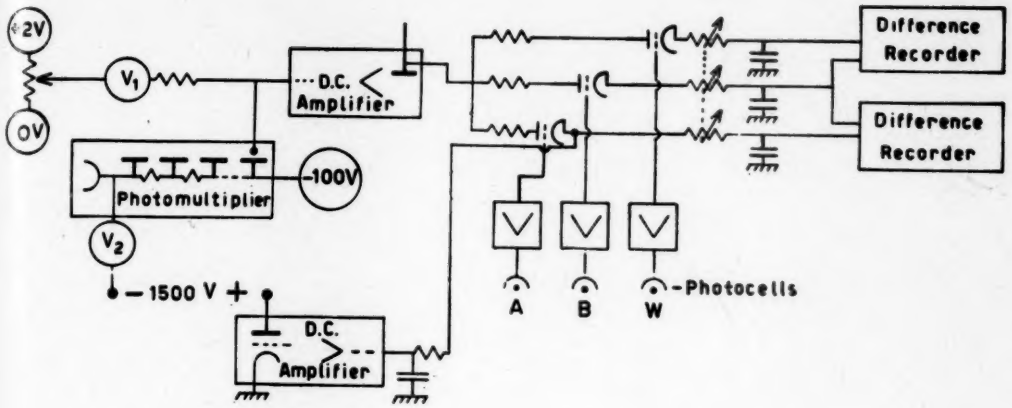
The diagram in figure 3 shows that during each cycle the photomultiplier is shut off 4 times instead of once by the fast disc. This is immaterial, since the 3 observations are made in between these closures.

The arrangement with long dark periods allows, firstly, to measure reversible (or step-wise) absorption changes by comparing signals B and A—at all times equal, if no actinic illumination is given.

Secondly, a comparison between signals W and B yields information concerning the absolute absorbance level at the end of the dark periods and thus indicates the density background (eventually also changed in the light), on which "fast" changes, A minus B, are superimposed.

By repositioning the 3 triggers, one can, if desired, also measure, for instance, A−W and B−W simultaneously. However, the 3 channels and the 2 recorders are closely alike, so that quantitatively (B−W) + (A−B) = (A−W) and 2 measurements are sufficient.

Measurement A−B concerns such fast changes that, compared to the duration of the individual effects, the integration and the response time of the recorder is inherently slow and therefore rather immaterial in any aspect other than accuracy. But in several instances measurement B−W (and also AB−W in the 2 channel set-up) indicates background absorption changes with time courses of the order of seconds. To study these, a rather fast response of the apparatus (down to 1 second) is advantageous. In this case such a fast indication is unobjectionable since the sensitivity of the method is mainly limited by differential fluctuations between the 2 beams (the V_2 compensating device, of course, can sense only 1 of the channels). Such fluctuations are not statistically random and little is gained by prolonged integration periods. (This is in contrast to the A−B measurements made with a single beam, which gain considerably in accuracy by prolonged integration).



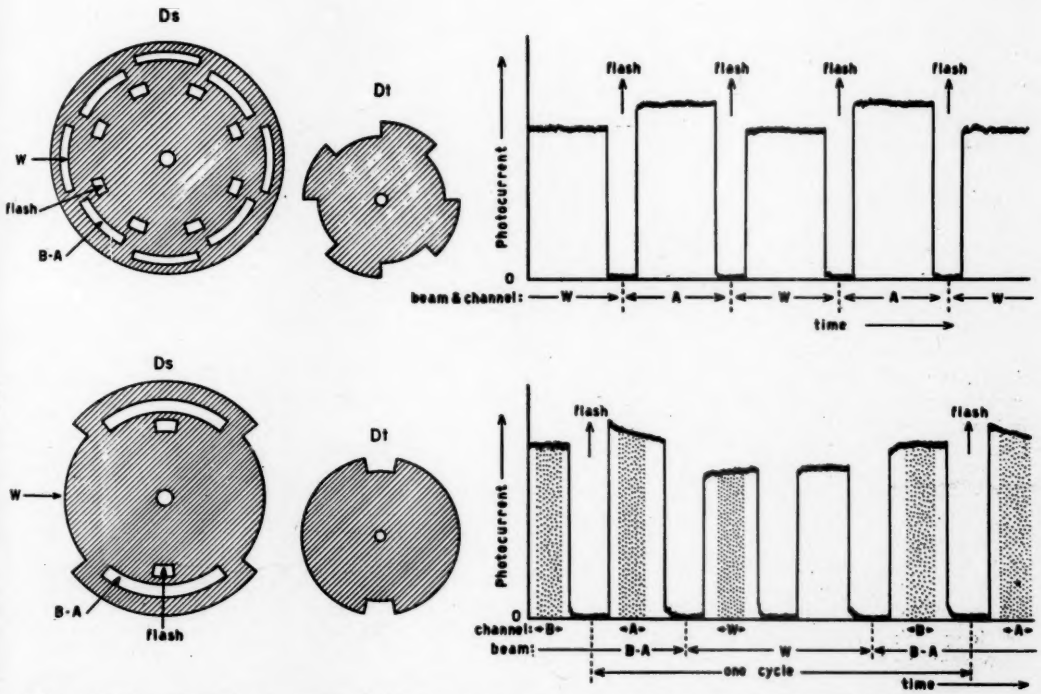


FIG. 2 (*top*). Simplified arrangement for the amplification, selection and recording of the photomultiplier signals. See text for discussion.

FIG. 3 (*bottom*). Illustration of the time courses of photocurrent (right) obtained with the "short dark time" arrangement (top) and the "long dark time" arrangement (bottom). The shapes of the slow disc (D_s) and the trigger disc (D_t) are also illustrated for each set-up (left).

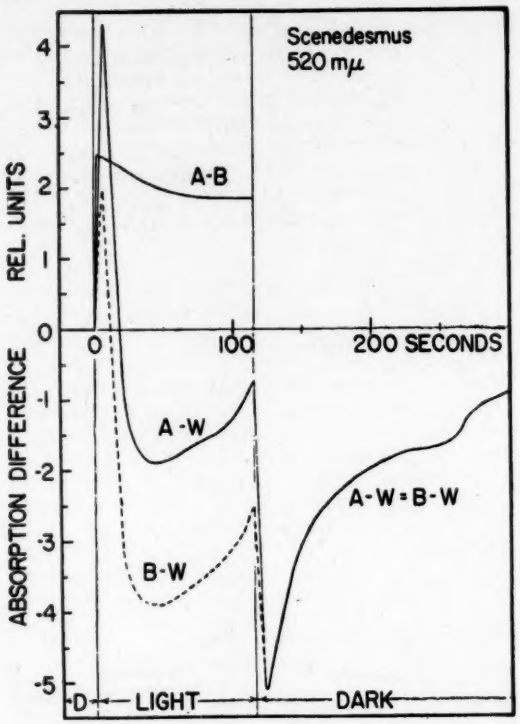


FIG. 4. Time course of signals A—B, B—W and A—W, as measured in 2 parallel experiments differing only in respect to the positioning of the trigger photocells (signal A—W therefore was directly measured rather than computed as the difference between the 2 other signals). Scenedesmus, I_o = 520 mμ. White actinic light.

EXAMPLES OF APPLICATION: As already mentioned, our main purpose for the described apparatus was a more intensive study of phenomena occurring in the red wave length area. Most likely, these bear closely on the primary photosynthetic events. Few investigations concerning these events have been made so far and these led to either negative or inconsistent results (1, 3, 11, 12, 14). Our first experiments with the apparatus described were made with the short dark time arrangement. The effects obtained did not agree with our earlier published data concerning fast effects. Subsequently therefore, mainly the 3 channel, long dark period method was used in studies of simultaneous rapid reversible effects and background changes.

The data of figure 4 illustrate that the complexity of the effects encountered, rather than methodological pitfalls often are responsible for apparently incongruent results. These data were chosen because they concern the extensively studied effects occurring around 520 mμ in Scenedesmus (3, 11, 12, 14, 18). The peculiar time course of the background curve (B—W), i.e., the absorbance level some 0.06 seconds after each light flash, closely matches data obtained by Strehler and Lynch (4) using a different method. On the other hand, in accord with our own earlier findings, the magnitude of the fast changes (A—B) is entirely unaffected by these seemingly drastic changes of background absorption (in fact all effects are within a range of only a fraction of a percent transmission change). Apparently the individual light flashes produce a constant amount of the molecular species involved. During the 1st 10 seconds of illumination, its formation is faster than its removal during the dark periods. After this induction period the rate of removal in the dark increases and temporarily surpasses the rate of formation so that at any moment of the flashing light cycle absorption is lower than the dark level. In contrast, our short dark time method showed—in accordance with other observations made in continuous actinic light—an increase of absorption in this wave length region.

Another peculiar phenomenon—of which one should be constantly aware in this type of measurement—is demonstrated in figure 5. The data, obtained with *Rhodospirillum rubrum*, show that at both wave lengths studied, a decrease of the intensity of the detecting beam (I_d) considerably increases the magnitude of the background absorption change (B—W) induced by the flashes. A 3-fold difference is observed in these particular examples. In other cases, we have observed both larger and smaller antagonistic effects between actinic and detecting lights. In the next section we will mention an observation of the reversed effect, i.e., the requirement for strong detecting light to obtain significant actinic light action.

Noteworthy in figure 5 is the observation that the magnitude of the fast changes (A—B which appears superimposed on B—W in curve A—W) is unaffected by the intensity of I_d. Also the fact that deflection B—W is at least equal to deflection BA appears significant (cf. below).

Figure 6 gives another example of difference spectra, each set of which was measured in 2 experimental runs with parallel samples of bacteria: 1st A—B together with B—W, 2nd A—B concurrently with A—W. The particular curves measured with *Rhodospirillum rubrum* do not show any special effects and are in general accord with Duysen's data obtained with continuous illumination (2). The 3 different curves follow a closely similar pattern, which indicate that the flash-induced changes revert in the dark, but not completely. Obviously, in the steady state flashing light pattern a considerable background concentration of converted pigment persists. Actually, as will be described in a later paper, the same situation seems to hold for nearly all the absorption shifts, which occur so abundantly over the range between 400 and 850 mμ in purple sulfur bacteria.

An interesting phenomenon in figure 6 can be observed in the region around 885 mμ in the spectra measured with Chromatium. Here both the A—B and A—W curves show a distinct band of absorption decrease, which, however, is absent in the B—W

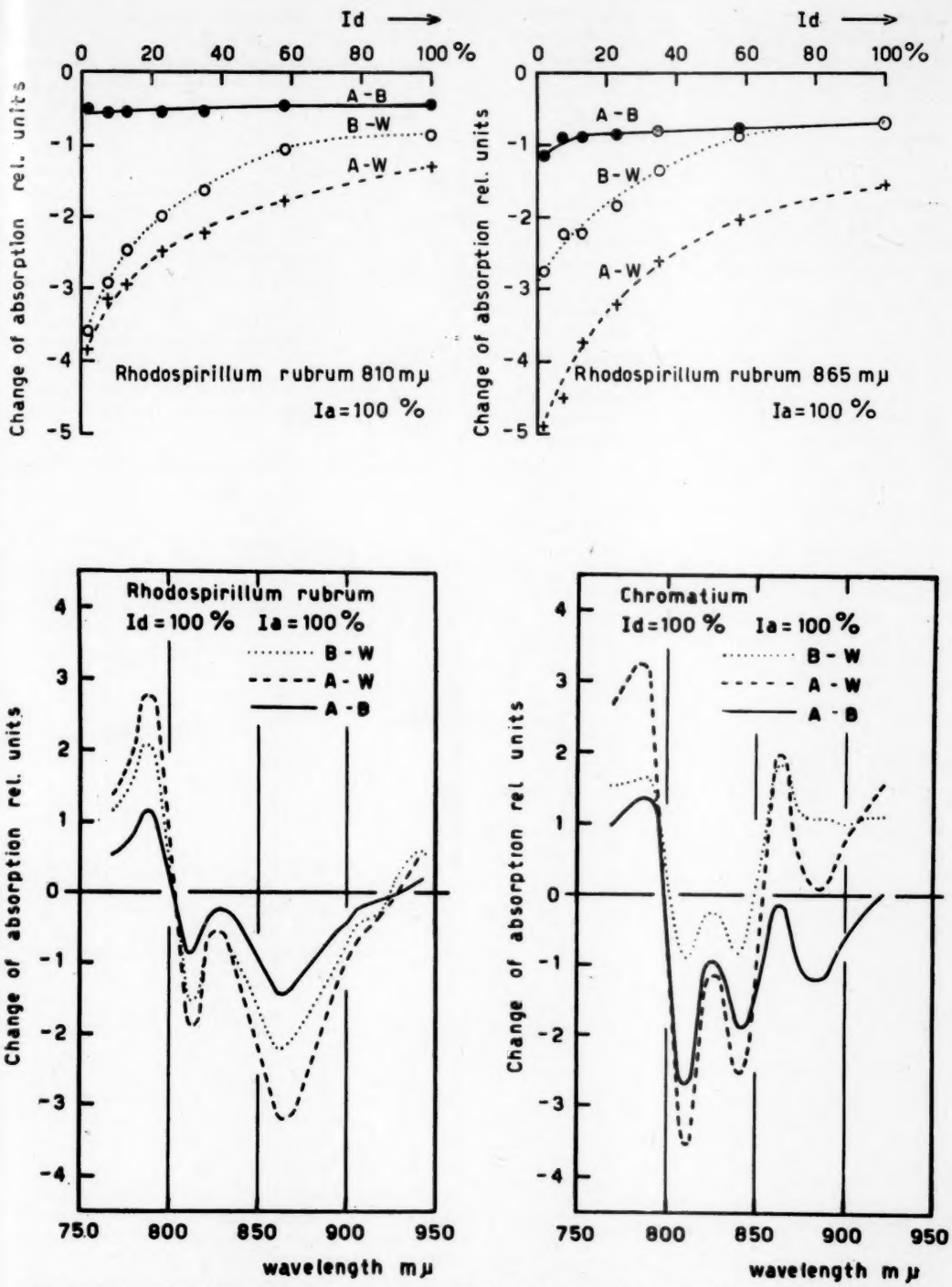


FIG. 5 (*top*). Dependence of the magnitude of signals A—B, B—W and A—W (all 3 measured in 2 experimental runs) upon the relative intensity of the detecting beam.

FIG. 6 (*bottom*). Difference spectra as measured in the infra-red with purple sulfur bacteria. Actinic light: 500 to 700 mμ obtained from a filtered mercury arc.

spectrum. This and related observations in photosynthetic bacteria seem to show basic similarities to the phenomena touched upon in the next section.

A Red, Far-red Antagonism in Photosynthesis: Partly to illustrate the versatility of the apparatus we may briefly describe a few observations made with the blue green alga, Anacystis. This alga was chosen as a subject of study because of its ready growth, its small size, which makes settling in the sample tube less of a problem, and because in our earlier work (11) blue green algae showed a quite pronounced reversible decrease of absorption around 705 mμ.

The phenomena observable in the red part of the spectrum appear to be extremely complex and the data to be described represent only the first steps towards a complete analysis.

We will restrict ourselves to some observations made in the wave length region between 690 and 720 mμ. Ribbon filament (6 V, 18 A) incandescent lamps were used both for the actinic and the detecting beam, a 1 cm water filter containing a trace of copper sulfate was at all times in the actinic beam.

Figure 7 shows a time course of the difference B−W measured with I_d = 703 mμ by alternately illuminating the sample with either white (non-filtered incandescent) or far-red light. A Schott RG 1 ($\lambda > 600$ mμ) filter in the actinic beam hardly decreased (↘ 30 %) the white light effect. Far-red light was obtained by placing a Schott RG 5 ($\lambda > 670$ mμ) in the beam; a Schott RG 8 or filter ($\lambda > 695$ mμ) yielded some 40 % smaller deflections.

The data in figure 7 indicate that far-red light yields a decrease of background absorption whereas red or white light has the reverse effect. The white or red light actually contained at least as much far-red radiation as was transmitted by the RG 5 filter. Obviously therefore the effectiveness of the 600 to 670 mμ radiation is predominant in this mixture. Also shown in this figure (dashed curve) are the deflections A−B caused by the respective illuminations. Note that these are negative, regardless of color, only the magnitude varying. The A−B deflections (fast reversible changes) are smaller than the B−W changes. So far we have often been able to observe exact equality of the 2 deflections and when unequal, A−B never exceeded B−W (disregarding signs of the changes).

In the case that deflection B−W is exactly equal and opposite to deflection A−B, (illustrated in fig 9), we meet the interesting case that the flashes destroy exactly as much "700 pigment" as is built up again in the dark period (A−W = 0). This pigment (or at least this active fraction) thus might not be present in the dark before the actinic illumination, neither may it become apparent under steady state illumination.

In this type of experiment the white light intensity required for building up the 700 pigment is quite low. Our earlier work showed that saturation of the fast effects (A—B) required intensities of the order of

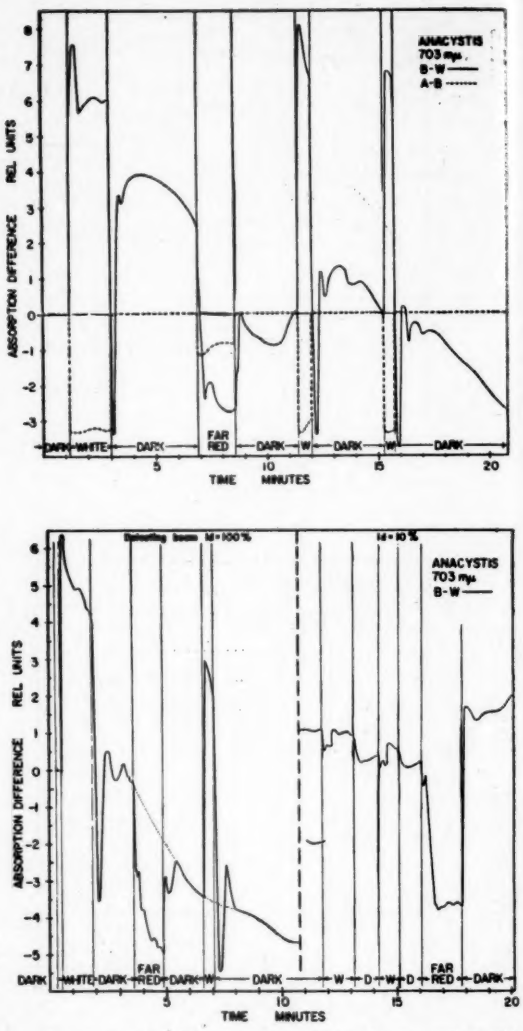


Fig. 7 (*top*). Time course of signals B−W (solid line) and A−B (dashed line) as observed with Anacystis cells alternately exposed to either white or far-red flashing light. Note the occurrence of transitory phenomena in many instance, and of slow absorption decay in the "dark" induced by white illumination. The full intensity of the detecting beam was used. The A−B deflections are negative in all instances.

Fig. 8 (*bottom*). Left: Similar experimentation as described in the legend of figure 7 except for omission of signal A−B. Right: Continuation of the experiment after the detecting beam was decreased to 10 % of the original value: effects in white light tend to disappear.

those needed for photosynthetic flash-saturation. But half-saturation of the background increase was already obtained at intensities some 3 to 10% of the available actinic light. Such intensities yield barely observable fast (A—B) effects. Even 100 % of the used incandescent illumination is rather weak in terms of photosynthetic activity. We tend to ascribe this low intensity requirement to the fact that a counteraction between detecting and actinic beam is involved. The detecting beam in itself (cf. e.g., figs 5, 7, 8) is strong enough to evoke considerable effects, observable as strong drifts of absorption, dependent in sign, magnitude and duration upon wave length setting and prehistory of the sample.

On the other hand, as is illustrated in figure 8, we also found that in extremely weak 705 mμ detecting light, the (B—W) red effect may disappear completely, whereas the far-red effect may be either unaffected or considerably increased. Apparently, since beam I_d does not cause appreciable breakdown, red light cannot further increase the concentration of 700 pigment, but far-red light finds abundant material to convert. However, since the A—B changes are hardly affected by a decrease of I_d, we obtain the reverse situation as was met before: instead of A—W being zero and B—W positive, now B—W is zero and A—W is negative. Generally, the relative background concentration of the 700 pigment indicated by signal B—W will tend to attain an intermediate steady state level, determined by the prevailing conditions, often preceded by transitory adjustments.

Figure 9 finally illustrates the close similarity of peak configuration—regardless of sign—of the 3 types of difference spectra.

The data discussed are far from exhaustive and our reasoning is necessarily over-simplified. The mere fact that a light flash photo-chemically destroys the pigment, but at the same time induces its reformation in the following dark period, indicates that dark phenomena (and therefore e.g., temperature effects) must be involved.

The described type of mechanism shows obvious similarities to the light effects encountered in plant growth regulation and might well be of more general significance in photochemical conversion by plants (7, 16, 17).

A significant interpretation of the described phenomena can be given only because of fundamental discoveries of Emerson and co-workers. In 1941, Emerson and Lewis (9) found in Chlorella a severe drop of the photosynthetic quantum yield at wave lengths beyond 680 mμ. This same phenomenon was observed in red and blue green algae (4, 8) as well as in Diatoms (11). Later, an influence of temperature upon the yield and long wave length-limit of photosynthesis was observed. The most striking observation, however, was that this long wave length-limit could be shifted towards the far-red by the addition of a supplementary amount of short wave length light ($\lambda < 650$ mμ). The conclusion from these data was that excitation of chlorophyll a alone—

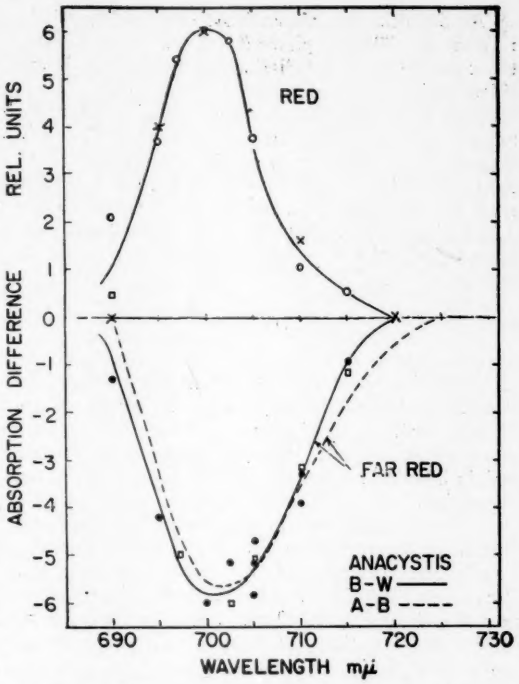


FIG. 9. Difference spectra B—W as induced by red light (incandescent with RG1 filter, open circles, crosses) and by far-red light (incandescent with RG5 filter, dots, squares). The dashed curve shows the A—B spectrum which was measured concurrently with the B—W observations indicated as dots. Curves are adjusted for equality of peak height.

although the only universally occurring photosynthetic pigment—does not yield efficient photosynthesis (6, 7).

In the light of Emerson's findings we can at least tentatively try to explain some of the effects we have observed. The location of the absorption band of the 700 mμ pigment—even if present in only a very small concentration—makes it ideally suited to effectively trap all the light which is absorbed by chlorophyll a itself, or transferred to it by accessory pigments (and which thus is transformed into "far-red" light). Suppose the 700 pigment is indeed the final light sink in photosynthesis and its excitation by surrounding chlorophyll a molecules leads to a conversion, which entails disappearance of its absorption. Then, from there on, the neighboring chlorophyll a molecules—say those comprising a "unit"—are deprived of their outlet until the 700 pigment is restored. This restoration, however, also requires light, but now only red and not far-red is active. Obviously, sensitization by chlorophyll a (which yields only 700 mμ fluorescence) cannot restore the bleached 700 pigment and photosynthesis cannot proceed effectively in far-red light alone.

As was described above, irradiation with red light restores the 700 mμ pigment and therefore sustains the conversion of irradiation transferred to it via chlorophyll a.

Further speculation, however tempting, does not appear to be fruitful at the present time. We would even have hesitated to presently venture the above thoughts if it were not to draw attention to a new road towards a better understanding of the most fundamental aspects of photosynthesis, opened up by the eminent scientist to whose memory this article is dedicated.

Summary

An apparatus is described, designed to register the small changes of absorption, which are induced by light in photosynthetic organisms. A split-beam arrangement allows the simultaneous observation of fast reversible absorption changes, caused by flashing actinic illumination and of slow shifts of background absorption.

A few examples of its application are discussed. One of these concerns the observation of an antagonistic effect between red and far-red light on Anacystis. It concerns the build up and break down of a pigment, normally absorbing at 700 mμ, present in low concentration, and tentatively identified as the end point of energy transfer in photosynthesis.

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INFLUENCE OF SELECTIVE LIGHT SCATTERING ON MEASUREMENTS OF ABSORPTION SPECTRA OF CHLORELLA[1, 2]

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Experimental information about the light scattering characteristics of biological cells is surprisingly scarce. Even though all measurements of absorption spectra of intact biological structures must be influenced to some extent by scattering, there have been few investigations dealing specifically with scattering by cell suspensions. However, recent experiments have revealed that light absorbing cells and cellular components in suspension scatter light with a strong spectral selectivity (5, 6). Scattering maxima occur on the long wave length sides at absorption maxima. Other studies have demonstrated that this selective scattering can influence measurements of absorption spectra, shifting apparent band positions by 10 to 15 mμ (7, 8). The present paper describes further investigations of phenomena related to selective scattering. We attempted to obtain information about light scattered at small angles to an incident beam from suspensions of *Chlorella pyrenoidosa* Chick (Emerson strain) by using special experimental methods to measure absorption spectra.

We have made use of the fact that the effects of scattering on measurements of absorption spectra of turbid cell suspensions depend on some of the experimental conditions. Specifically, we measured two series of absorption curves in which a single factor that influences the effects of scattering was varied throughout a series. In the first case, absorption curves of a cell suspension were measured with a special spectrophotometer which allowed the detector to view only that light coming from the cell suspension at certain predetermined angles to the incident beam. In the second series of measurements, we fixed the geometry of the spectrophotometer so that the light detector received only directly transmitted light (parallel to the incident beam) and varied the index of refraction of the suspension medium from curve to curve (see Barer (1)).

APPARATUS AND PROCEDURE

In order to measure absorption curves by allowing the light detector to observe only light coming from the suspension at specified angles, we constructed a special spectrophotometer by modifying the fluorescence spectrophotometer of French (4). The sample holder and mirror system was replaced by a tungsten light source, and baffles and a suspension vessel were inserted between the exit slit and the light detector (Photomultiplier tube, R.C.A. 6217) (see fig 1). The baffles isolated a parallel ($\pm$ 1°) beam of light from the monochromator output. A thin (1/8 inch) layer of suspension was used to reduce uncertainties in the angle of observation. A special baffle (angle selecting assembly) was constructed which alternately permitted the photomultiplier tube to observe 0° (directly transmitted) light (through the small center opening) or light scattered at an angle to the incident beam (through the doughnut shaped opening, the center of which was in line with the incident beam). The angle of observation could be increased by moving the angle selecting assembly (and the photomultiplier tube and diffusing plate) closer to the suspension vessel. The doughnut shaped opening was used instead of a simple small hole for defining the angle of observation at non-zero angles since it allowed more light to pass with the same angular resolution. The diffusing plate (made of opal glass) eliminated errors which might be produced by differences in the sensitivity of different parts of the photocathode.

As a control, we also measured absorption curves of the suspensions by placing diffusing plates at the suspension and blank vessels as suggested by Shibata (11). Since the effects of scattering on this latter curve were the least, and since this method has been found to yield absorption curves in general agreement with those obtained by the more reliable integrating sphere technique, which further reduces the effects of scattering (compare curves in references (5) and (7)), we assume that absorption curves measured in this way are good approximations of the actual absorption spectrum of the cell suspensions. For these measurements, the angle selecting assembly was removed, the diffusing plate was placed against the vessels, and the photomultiplier tube was moved to the left.

To determine the spectral absorption curves, we measured separately (using the automatic sweep mechanism) spectral curves of the light passing through the suspension (at a specified angle) and through the blank vessel (at 0°). Actually 1 curve was: (output of monochromator) $\times$ (sensitivity of light detector system) $\times$ (transmission of suspension) = $f(\lambda)$, while the other was: (output . . . .) $\times$ (sensi-

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[2] Parts of this investigation were assisted by a research grant, (RG-5516) from the National Institutes of Health, Public Health Service.

tivity . . . .) × (transmission of blank) = f′(λ). Since the first 2 terms in the products are the same (see below), "absorbance" curves were obtained by dividing the levels of the curves at 5 mμ intervals and changing to logarithmic coordinates (absorbance = log I_o/I).

The 2 types of curves were measured alternately, 3 sweeps of the spectral region investigated being made in each case. The averages of the 3 curves of each type were used to calculate the curves shown in figure 3.

This method requires that the product, (output of monochromator) × (sensitivity of detector system), remain constant during the entire determination of 1 curve. This condition was usually fulfilled as evidenced by agreement between different curves of the same quantity taken at different times. When such curves disagreed, the data were discarded.

Absorption curves of cells in suspension media of different indices of refraction were measured with a Beckman DK-2 recording spectrophotometer. The normal arrangement of its optical system was used to measure directly transmitted light (0°±3°) (arrangement (a) in fig 2), while diffusing plates were placed after the suspension and blank vessels (against the vessel holder) for the 0° to 90° curves (arrangement (c) in fig 2). Further details of this method are given in references (7) and especially (8). Media of different indices of refraction were prepared by dissolving bovine serum albumen (Armour's fraction V) or glycerol in water (buffer). Cells from the same culture were added to each medium to yield suspensions of equal cell density.

Experimental Results

Dependence of Absorption Spectra on Geometry of Optical System: Experimental absorption curves in which the angle of observation of the light detector was varied are shown in figure 3. The upper curves are absorption spectra measured with the photomultiplier tube observing only light coming from the suspension at 0°, 3.4°, and 32° respectively. The curves are quite different, not only in shape but also in the position of the "absorption" maximum (688 mμ at 0°, 682 mμ at 3.4° and 668 mμ at 32°). Other measurements show this shift to be a gradual one with angle. Although we were not able to extend the measurements to larger angles with this apparatus, the results of our previous measurements of light scattered at 90° are very nearly comparable (5, 6).

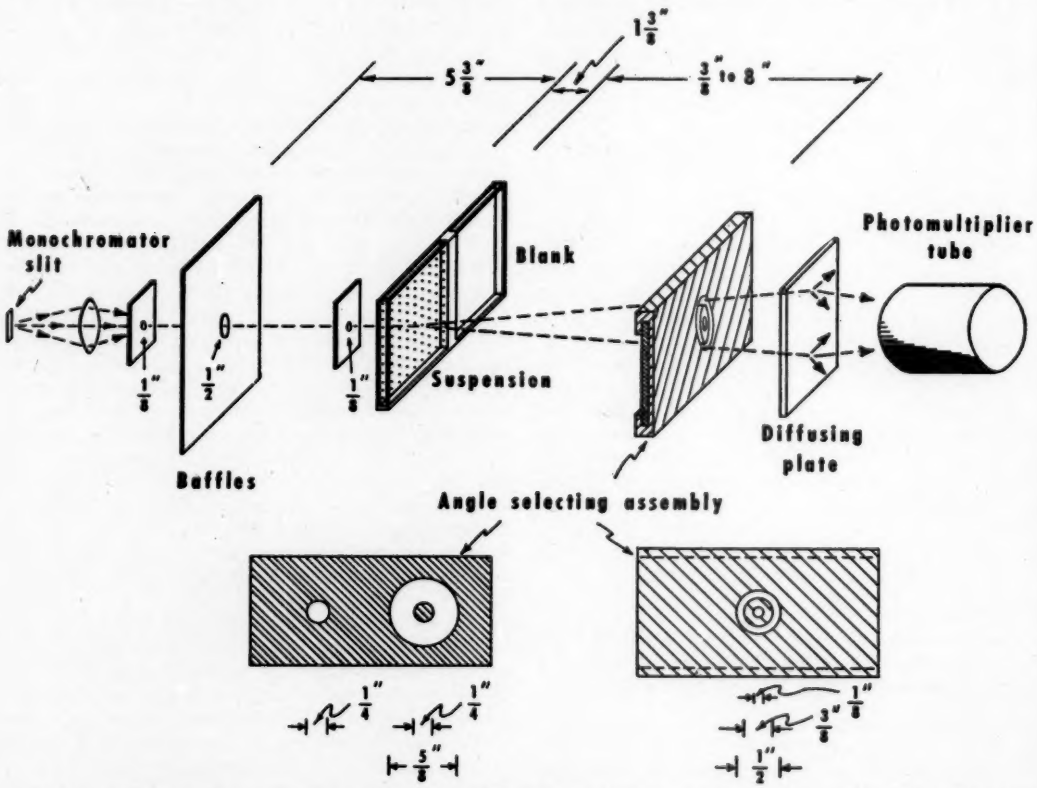


Fig. 1. Apparatus for measuring spectral curves of light transmitted or scattered at small angles to the incident beam. The angle is varied by changing the distance between the suspension and the angle selecting assembly.

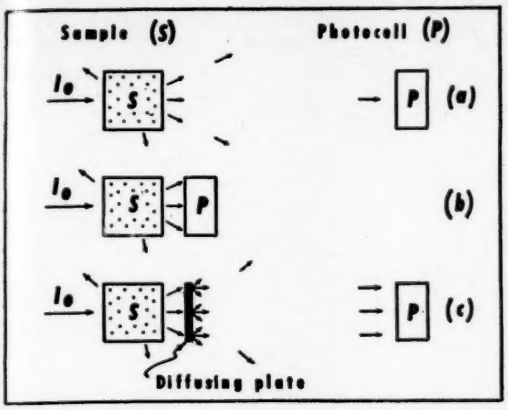


FIG. 2. Schematic diagrams of part of the optical system of a spectrophotometer.

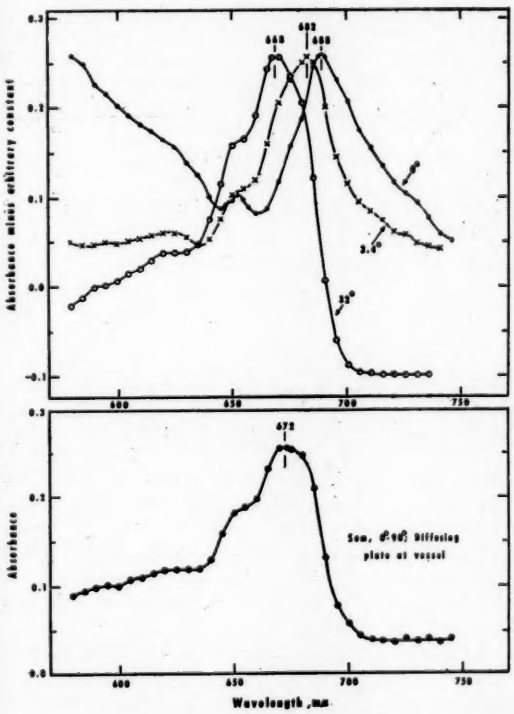


FIG. 3. Chlorella: upper curves, absorption spectra determined by measuring light transmitted or scattered by a cell suspension at indicated angles with the apparatus in figure 1. The curves were arbitrarily normalized in height. The indicated angles, 0°, 3.4°, and 32°, are not corrected for refraction at the vessel surface. This correction changes the angles to 0°, 2.6°, and 24° respectively. The lower curve was determined with the diffusing plate against the vessel, as in Figure 2 (c).

If the curve for 90° scattering by Chlorella were plotted as an absorption curve (this would require inverting the curve and changing from linear to log units), the "absorption" maximum would appear at about 655 mμ which is still further to the left.

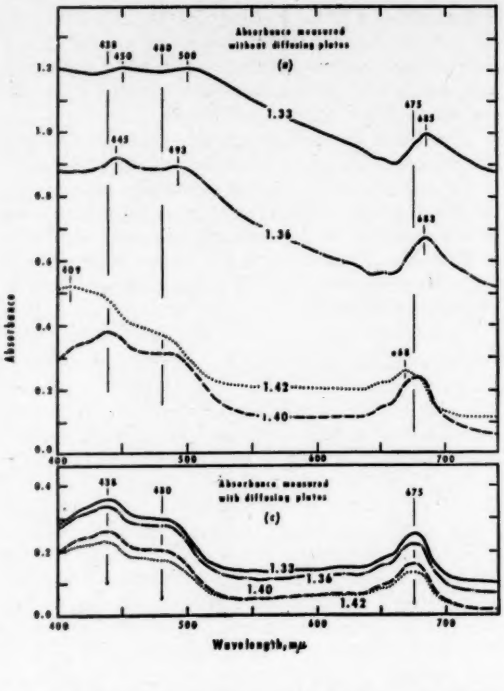
The lower curve in figure 3 (maximum at about 672 mμ) was obtained by placing the diffusing plate at the suspension vessel. It essentially represents a weighted average of spectral curves (such as those shown above it) for light coming out of the suspension at all angles of from 0° to 90° to the incident beam and with absorption maxima varying in position from 688 mμ to about 655 mμ.

DEPENDENCE OF ABSORPTION SPECTRUM ON INDEX OF REFRACTION OF SUSPENSION MEDIUM: Absorption spectra of Chlorella in media of different indices of refraction as measured with the Beckman DK-2 spectrophotometer (using arrangements (a) and (c) in fig 2) are shown in figures 3 and 4. First, it should be noted that the positions of the absorption bands of all suspensions (except that for medium, n = 1.40) are different in the upper and lower series of curves (see reference (7) for a preliminary report of this effect). For instance, the red absorption maximum of cells in water (n = 1.33) is at 685 mμ in the (a) series of curves while it occurs at 675 mμ in the (c) series.

As the index of refraction of the medium is increased towards approximately that of the cells, assumed here to be 1.40, not only does the general height of the curves decrease, but the apparent positions of the bands on the upper curves shifts towards the actual position of the bands. On further increasing the index of refraction of the medium to 1.42 (approximately 40 % albumen), the shift to the left of the band positions continues so that the red band now appears at 668 mμ.

It should be noted that the positions of the bands on the lower curves in figure 4, which presumably represent the best approximation of the actual absorption spectra, are not significantly altered by the medium. Therefore, the variations in the positions of the bands in the upper part of the figure must be attributed to optical phenomena and not to actual differences in the true absorption spectra of the cells.

On the other hand, a similar series of measurements on Chlorella cells in glycerol-water solutions gave quite different results (see fig 5). While the general level of the upper curves is reduced somewhat as the index of refraction of the medium increases, only a slight shift in the apparent positions of the bands is observed. Other curves in the same series for intermediate values of n were similar to those shown in the figure. The general difference between the behavior of the curves in figures 4 and 5 can be attributed to differences in the nature of the additives, bovine serum albumen and glycerol, respectively. The albumen, being a large molecule, does not penetrate the cell membrane, and the osmotic pressure of its solutions is



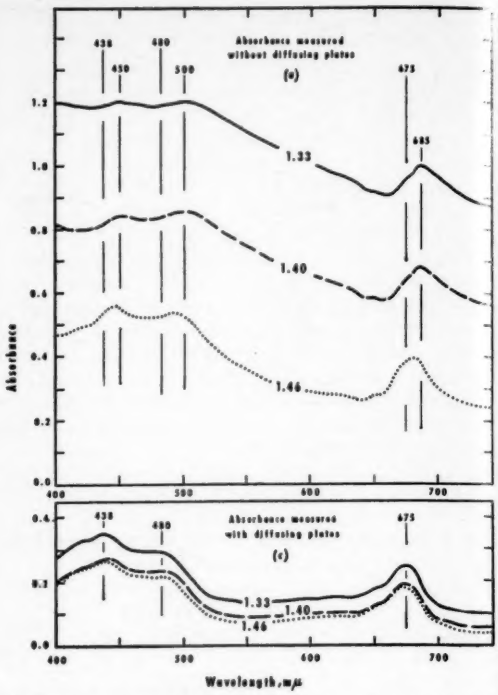


FIG. 4 (*left*). Chlorella: absorption spectra of suspensions of equal cell density in different water solutions of bovine serum albumen having the indicated refractive indices. The curves were measured with a Beckman DK-2 recording spectrophotometer using arrangements (a) and (c) in figure 2.

FIG. 5 (*right*). Chlorella: absorption spectra of suspensions of equal cell density of water-glycerol solutions having the indicated refractive indices.

probably not very different from that of water. However, the small glycerol molecule readily penetrates the cell membrane and the osmotic pressure of its solutions is high. Hence, the optical properties of the cells in glycerol-water solutions must be different from those of cells in water or albumen solutions because of penetration of the glycerol and the exit of water from the cell. Both processes increase the index of refraction of the cells. Scattering depends on the difference between the index of refraction of the cells and that of the surrounding medium (see next section). It is suggested that the addition of glycerol to the medium did not effect large changes in this difference since both indices are raised by it.

As noted elsewhere (8), the positions of the maxima on curves measured with arrangement (a), figure 2, vary slightly with suspension concentration (presumably because some of the observed light has encountered 2 or more cells). For cells in water, the thinnest suspensions gave peaks displaced furtherest to the right. We estimate that on extrapolating to zero concentration, the band maximum at 685 mμ in the upper part of figure 4 would be shifted to about 689 mμ.

THEORETICAL INTERPRETATION

Many similarities exist between the angular dependent curves in figure 3 and the medium dependent curves in figure 4. In both cases, the position of the "absorption" maximum is found on either side of the actual maximum depending on experimental conditions. Both sets of results are clearly related to the phenomena of selective scattering which can be explained in terms of the theory of scattering by absorbing particles (9).

To a first approximation, total scattering by both absorbing and non-absorbing particles depends on the index of refraction (relative to the surrounding medium) of the scattering particle according to the following equation:

$$S = k\,(m-1)^2 \qquad (1)$$

where S is scattering per unit incident light, k is a constant and m is the relative index of refraction of the particle. (If the index of refraction of cells is 1.40 and that of the medium (water) is 1.33, the relative index of refraction of cells is 1.40/1.33 = 1.05.) According to the theory of anomalous dispersion, m

becomes a complex number in regions of absorption ($m = n - in'$ where we call n the real part and n′ the imaginary part). Both parts vary rapidly and in a characteristic manner with wave length. The imaginary part of m is very nearly proportional to absorption while the real part varies assymetrically about the absorption band. (See figure 6 A for theoretical curves of these 2 parts for Chlorella.) Since the real part of m passes through a maximum on the long wave side of the absorption band, S (in equation 1) should also pass through a maximum on the long wave length side if $m > 1$. This effect which we called selective scattering, was observed in studies of 90° scattering of several different biological systems.

It would be desirable in this section to use an exact theory of scattering by colored particles to calculate theoretical curves for comparison with all of our experimental curves. While it appears that the Mie theory of scattering by light absorbing particles should be capable of explaining all of our results, the calculations for absorbing particles of this size would be almost prohibitively complex. On the basis of limited efforts to apply the theory to Chlorella, we estimate that the determination of a single point on one spectral curve would involve several thousand calculations.

On the other hand, van de Hulst (12) has obtained approximate expressions from the rigorous Mie theory and the classical theory of dispersion which predict extinction and absorption by some homogeneous spheres. The equations, as restated in reference (9), are useful in predicting extinction and total scattering by nearly spherical biological cells. We used it here, in a further modified form which eliminates some of the approximations, to calculate extinction curves for Chlorella cells in media of different indices of refraction. Since the upper curves of figure 4, for Chlorella in bovine serum albumen solutions, are actually extinction curves (extinction = absorption + total scattering), the theoretical curves should agree reasonably well with these experimental curves if, indeed the theory is applicable to suspensions of Chlorella cells.

A Chlorella cell was assumed to be a homogeneous sphere, diameter: 3.2 μ. We assumed its average index of refraction at wave lengths remote from absorption bands to be 1.40. The index in regions of absorption was calculated from the classical theory of dispersion using the extinction coefficient of cells (see reference (9)) and a band half-width of the red absorption band of 33 mμ. The absorption curve was assumed to be symmetrical about 675 mμ. No allowance was made for the shoulder at 650 mμ which is presumably caused by chlorophyll b.

The theoretical extinction curves are shown in

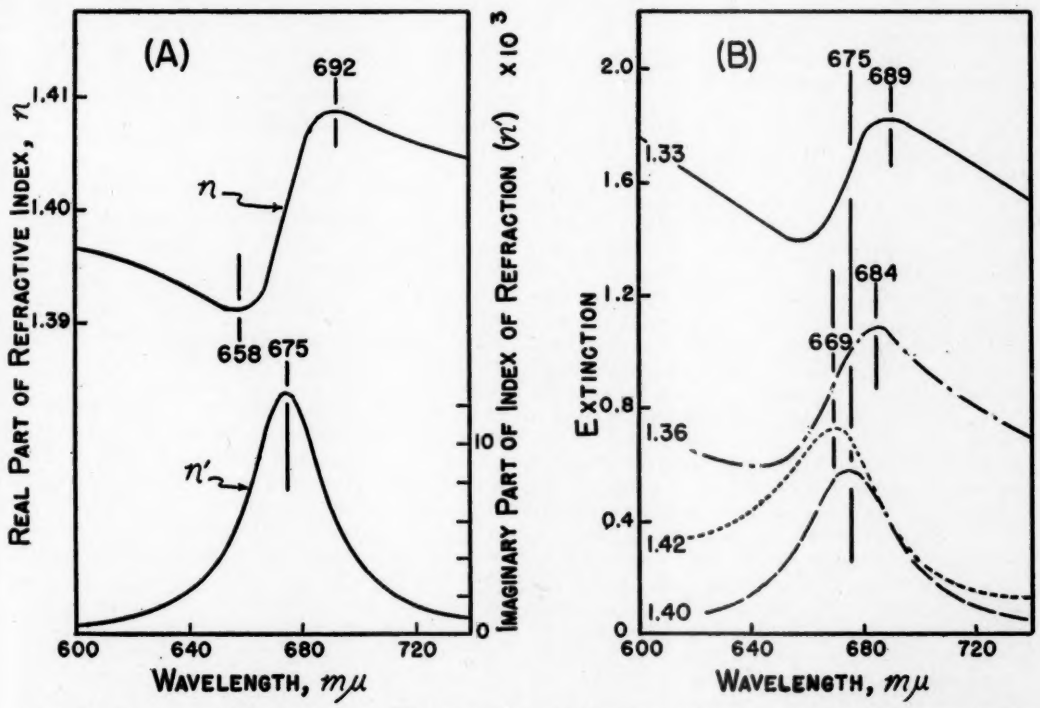


FIG. 6. Chlorella: theoretical curves. (A) Real (n) and imaginary (n′) parts of average index of refraction of entire cell (2). (B) Extinction of cells in media of indicated refractive indices, calculated using (A) above and (12).

figure 6 B. Comparison with the corresponding experimental curves in the upper part of figure 4 reveals that the observed dependence of the band positions on the index of refraction of the medium is clearly predicted by theory. The bands on the theoretical curves at 689, 684, 675, and 669 mμ are in good agreement with those on the corresponding experimental curves at 685, 683, 677, and 668 mμ respectively. It is likely that the discrepancy between band positions, 689 and 685 mμ for cells in medium, n = 1.33, is caused by effects of multiple scattering on the experimental curve as previously mentioned. The difference between the band positions of 677 and 675 mμ for cells in a medium of n = 1.40 may be caused by our assuming for the theoretical calculations, a value (1.40) of the index of refraction of the cells (at wave lengths remote from the absorption band) which is too low.

On the other hand, the general levels of the experimental curves vary more with the index of refraction of the medium than do those of the theoretical curves. This difference between experiment and theory may be caused by the following factors. The logarithmic ordinate scale was used for the experimental curves while the scale for the theoretical curves is linear. Furthermore, thus far we have made no allowance for effects of scattering by non-absorbing components of the cells. While it is difficult to say how the omission of this factor would influence the results, it might easily produce the above mentioned discrepancy between experiment and theory.

There is also another less rigorous, but more tangible, theoretical explanation of the upper curves in figure 4. It might be suggested that the displacement of the absorption band of Chlorella in water (when measured at 0°) from its actual position at 675 to 685 mμ can be explained in terms of the fact that we actually measured extinction which is the sum of absorption plus total scattering. Thus if the actual absorption band lies at 675 mμ and the scattering band at 690 mμ (see reference (5)), a suitable additive combination of the 2 bands would yield an extinction band at 685 mμ. As one increases the index of refraction of the medium (to 1.36 and 1.40) toward that of the cell (about 1.40), the magnitude of scattering (see equation 1) and its contribution to the extinction band is reduced, and the band is shifted from 685 to 683 mμ to about 675 mμ. Now, passing on to a medium (n = 1.42) of index of refraction greater than that of the cells, we see that (m−1) in equation 1 becomes a negative number (although $(m-1)^2$ will nevertheless be positive). In this case, the index of refraction of the cells (see fig 6 A) has a minimum on the short wave length side of the absorption band, so that the quantity (m−1) has a minimum value since m < 1 but nevertheless $(m-1)^2$ has a maximum value. In this case there should be a scattering maximum on the short wave length side of the band instead of the long wave length side. Thus the extinction would be the sum of the absorption (maximum at 675 mμ) and scattering (peak between about 659 and 675 mμ). And indeed, the maximum extinction does seem to represent a sum of 2 such components with a maximum at 668 mμ.

On the other hand, if we consider the upper curves in figure 3 for the angular dependence of the absorption, neither equation 1, van de Hulst's theory, or the above considerations are applicable, and no theoretical explanation is offered at this time. However, some understanding of the observed phenomena of angular dependence of band position may be obtained by considering that advanced by Lothian and Lewis (10) to explain the results of their spectral curves of red blood cells. These authors reported interesting extinction and absorption curves for red blood cells which were measured by methods similar to both of ours for Chlorella in water, n = 1.33 in figure 3. (Their spectral curves have been substantially reproduced under special conditions in our laboratory by J. A. McClure.) While their data might in part be interpreted in terms of a peak shift caused by selective scattering as we suggested above, they explained the differences in their curves in terms of interference and diffraction effects. The explanation involves interference between light passing through the cell with that passing around it.

We have previously described absorption spectra measured with a diffusing plate at the suspension vessel both as reasonable approximations of the actual absorption spectra and also as a composite of a number of different spectral curves such as those in figure 3. It doesn't seem immediately obvious how the diffusing plate curves can be these two things at once. However, the explanation appears to lie in the interpretation of the curves in figure 3 in terms of interference and diffraction effects.

Discussion and Conclusions

By selecting the experimental conditions for measuring absorption spectra of the green alga *Chlorella pyrenoidosa*, we found that the positions of the absorption bands vary with the conditions over rather wide wave length intervals. The positions of band maxima as well as the shapes of the absorption curves are functions of both the index of refraction of the surrounding medium and the geometry of the optical system of the spectrophotometer. These effects are strictly optical in nature and do not reflect actual changes in the absorption spectra of the cells, which according to curves measured with diffusing plates, remained constant through all measurements.

Actually it should be noted that the position of the red absorption band (672 to 675 mμ) as measured with diffusing plates in figures 3 and 4 is slightly different in the 2 cases. The cells used in these 2 measurements were from different cultures. We believe that this difference is a real one although it might be accounted for in other ways. For instance, different spectrophotometers were used for the 2 sets of measurements and there may have been slight calibrational errors. Also, the diffusing plate does not collect all scattered light from the suspension but only that

emerging in the forward direction. The level of curves at 740 mμ where little if any absorption occurs is probably caused by loss of light caused by back scattering. It is possible that differences in the scattering characteristics of the cells from different cultures so effected the back scattered light that the apparent band position was shifted.

It may well be that many of our curves measured in special ways (upper parts of figs 3, 4, and 5) should not actually be called absorption spectra. This illustrates the fact that while absorption spectra of non-scattering dyes in solution have a rigorous operational definition, no counterpart exists for turbid suspensions. However, in normal measurements of absorption spectra of biological cells and structures, we attempt to obtain curves which approximate those which would be obtained if the light absorbing compounds, in the same physical and chemical state, were uniformly distributed in a medium such that no scattering occurred. Unfortunately, these experimental conditions cannot be attained even in an integrating sphere which detects a representative sample of all light scattered out of the suspension vessel. While the integrating sphere appears to provide the most reliable measurements of absorption spectra, results obtained with it, as well as by most other methods, are nevertheless influenced by second order effects of scattering within the suspension (such as alteration of the effective path length of light through the suspension). Another factor which distorts absorption curves is the mutual shading effect which has been described theoretically for non-scattering particulate suspensions by Duysens (3) and E. E. Jacobs (the effect flattens absorption bands).

The optical effects reported here, and also those mentioned above, point to the fact that the events which occur when a beam of light enters a suspension of biological cells are still poorly understood. It appears at the present that instead of attempting to devise, by empirical means, a better experimental method of measuring absorption spectra of cell suspensions, it is more appropriate to first gain a clearer understanding of the events which occur on the passage of light through the suspension. With this, perhaps we may be in a better position to intelligently interpret the results of any spectroscopic measurements on turbid systems.

Summary

The scattering of light by *Chlorella pyrenoidosa* was investigated by measuring absorption spectra as influenced in different ways by scattering. Two series of measurements were carried out. In one case the cells were suspended in buffer solution and absorption spectra were measured with the light detector observing only that part of the total transmitted or scattered light coming from the suspension at well defined angles. In the other case, the cells were suspended in media of various indices of refraction and absorption spectra were measured by allowing the light detector to observe only directly transmitted light. The apparent positions of the absorption bands were found to be strong functions of both the angle of observation of the light detector and the index of refraction of the medium. The latter results were found to agree well with the predictions of scattering theory.

The vessel and baffle assembly in figure 1 were constructed by Mr. Richard Hart, who also drew most of the figures. Messrs. J. A. McClure and C. W. Hamlet performed some of the calculations. We also wish to thank Drs. C. S. French and J. H. C. Smith and Mr. H. W. Milner for their many helpful suggestions. It is appropriate at this time to point out that our study of light scattering by cell suspensions, which began when the author was a student at the Photosynthesis Project of the University of Illinois, was first suggested by the late Dr. Robert Emerson.

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CHROMATIC TRANSIENTS IN THE PHOTOSYNTHESIS OF A GREEN ALGA [1]

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Characteristic variations of O_2 production have been observed on changing the illumination of photosynthetic tissue from one wave length to another, even though the steady state of photosynthesis became the same in each case. These were designated "chromatic transients" (1). They were first found in red algae (e.g., Porphyra) where they amounted to some 10 to 15 % of the steady rate, on alternating the illumination from 675 mμ (chlorophyll a absorption peak in the living tissue) to 540 or 560 mμ (phyco-erythrin maxima).

These transients were at first thought to be absent in green or brown algae, but have now been found in comparable magnitude in the marine green alga, Ulva. These will be described in the present paper, which is dedicated to the memory of Robert Emerson, and his brilliant work on the role of various pigments in photosynthesis.

MATERIAL AND METHOD

The method was that already described (2, 9), utilizing polarographic or amperometric measurement of O_2 arriving at a bright platinum electrode, cathodally polarized at 0.5 volt. The potential drop across a resistance inserted in the circuit was tapped by a Speedomax recorder. The tissue, the marine alga *Ulva lobata* (Kütz.) Setchell and Gardner, was held tightly against the electrode by a strip of permeable cellophane. The thallus was only 2 cells thick, and the diffusion distance from the nearest cells to the electrode was only a few microns—the thickness of the cellulose wall. This gave a very rapid response to alterations of O_2 derived from the tissue. In the dark the current sank to a rather steady value within about 15 or 20 minutes of completing the circuit. This steady current was due to the O_2 diffusing from the sea-water, which could be aerated, or circulated. (In most of the present experiments it was equilibrated with 1 % CO_2 in air, to give an adequate carbon source.) Upon illumination from a large-aperture monochromator previously described (9) O_2 was photosynthetically produced and diffused to the electrode, where it was reduced by hydrogen ions, giving rise to an increased current. This time course may occupy 1 to several minutes, depending upon conditions. If the tissue had been dark for some time there were complicated induction phenomena, previously described (2). Even with fairly rapid alternations of light and dark, the time course was not always simple, and was sometimes a function of wave length; thus at 490 mμ there often was a cusp, (fig 1 a); this was smaller or missing at 540 mμ. To a lesser degree these differences occur (fig 1 b) at 640 and 688 mμ. But, just as in red algae, such second-order differences in the light-dark transients were often obscured by the time taken for the very large new diffusion gradients to be established. The chromatic transients became much more striking if dark periods did not intervene between exposures to the different colors of light, but if instead the tissue was allowed to attain a steady state of photosynthesis at 1 wave length, whereupon the wave length of incident light was abruptly changed (fig 2). Such transients amounted to some 10 or 15 % of the steady state level above the base line, and could be studied at considerably higher sensitivity by appropriate adjustment of the electrical circuit.

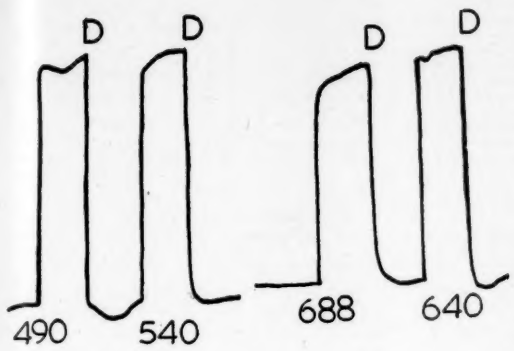
RESULTS

CAROTENOID TRANSIENTS: Data shown in figure 3 were obtained at greater sensitivity than those in figure 2. The tissue had been exposed to green light at 540 mμ. This was almost entirely absorbed by chlorophyll, probably about equally by chlorophyll a and b (based upon the pigments in solution). The tissue then was illuminated with blue-green light of wave length 490 mμ, adjusted to give as nearly equal rates of steady state photosynthesis as possible. This light was more strongly absorbed by carotenoids.

There was an immediate increase of current, giving a spike which lasted 30 seconds to a minute. This was followed by a decrease or depression, occupying several minutes. Then recovery occurred to the steady state originally obtaining. The time course of figure 3 rather closely resembles that shown in red algae on illumination with light absorbed by the phycobilin pigments (1).

On returning the illumination to 540 mμ (chlorophyll absorption), there was a simpler time course: a depression of current, followed by a slow recovery. These alternations of wave length gave rise to similar time courses as long as they have been studied—sometimes for several hours. If the steady state values were not exactly adjusted, one still obtained the characteristic cusps, depressions and recoveries, even though these may then be elevated or depressed from the base line. They may become even more conspicuous as the tissue ages, and greater post-illumination respiratory changes occur. The significance of these is referred to in the discussion. Smaller transients, of the same general shape, were found at lower light intensities (fig 3 a).

[1] Received March 24, 1959.



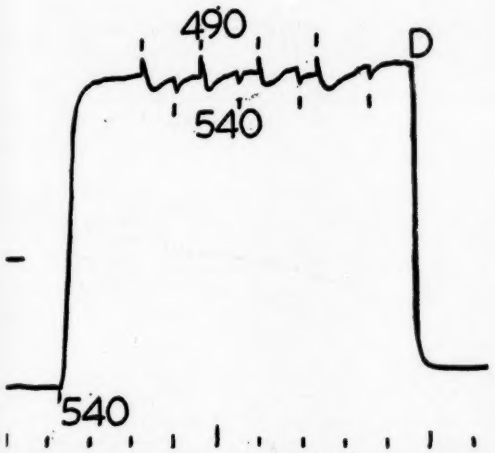


FIG. 1 (*top*). *Ulva lobata*. Tracings of responses to (a), left, 490 and 540 mμ, and (b), right, 688 and 640 mμ, of intensities adequate to give about equal steady state photosynthesis, but with intervening dark periods (D). There is a somewhat cusped time course in each case with the shorter wave length, and a depression of the base line below the steady dark value, just after illumination. This may indicate enhanced respiration at 490 and 640 mμ.

Recording rate as in figure 2.

FIG. 2 (*bottom*). Time course of photosynthetic oxygen production in *Ulva lobata*. The initial trace is in the dark. At 540, light of that wave length is given the tissue, and the current at the electrode increases due to O_2 evolution. When this has reached a steady state, the wave length is changed to 490 mμ, of intensity adequate to maintain the same steady state. There is a cusp, depression and recovery to the previous level. On return to 540 mμ, there is a depression and recovery. These exposures are alternated 3 more times, after which the illumination is stopped and the current returns to the dark level (D). Time marks at base, 10 minutes apart.

CHLOROPHYLL TRANSIENTS: If the wave lengths of incident light were alternated between 520 and 550 mμ (absorbed about equally by chlorophylls a and b, without carotenoid involvement) small transients occurred. Upon alternating between 666 and 686 mμ (wave lengths largely absorbed by chlorophyll a alone), there were also slight transients, upward at the shorter wave length, downward at the longer. It seemed possible that both cases might be due to the partial participation of chlorophyll b. Consequently the response was studied at 2 wave lengths predominately absorbed by chlorophyll a and b respectively. While it is somewhat uncertain exactly where the chlorophyll b absorption maximum may lie in the plant, 640 mμ was chosen for this region, with a corresponding steady state level at longer wave length, 688 mμ (a region of predominant chlorophyll a absorption).

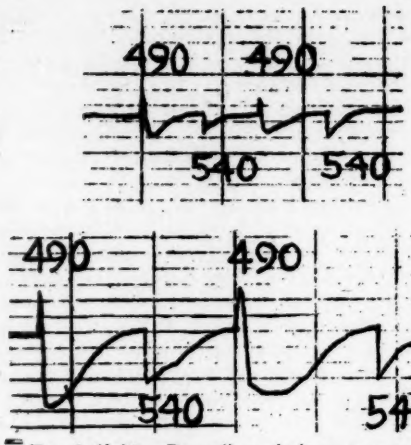
Here the transients became quite prominent, as shown in figure 4. The time course at 640 mμ resembled that at 490 mμ, although the cusp was sometimes shorter lived, and the succeeding depression less deep. The drop on returning to 688 mμ also was very sharp, and shorter lived.

DISCUSSION

What is the cause of these transient alterations in O_2 evolution, which result from successive illuminations by 2 different wave lengths of light? In the case of the red algae, a temporary "solarization" was suggested, by which the phycobilins in some way partially inactivate the chlorophyll a, as shown by the action spectra (9). Red algae also display "adaptation" in red light (11). The spike (a) in figure 5, in green light might represent an initially higher rate prevailing before inactivation, the valley (b) the depth of such inactivation, and the rise (c) some compensatory recovery phase, perhaps the generation of some new "active chlorophyll." The red depression (d) could represent the partial inactivation of chlorophyll persisting after the green light, from which there was slow recovery (re-adaptation in red light).

This explanation seems hardly likely in the green algae, where no evidence of such a pigment competition is at hand. Conceivably the effect is due to partial bleaching and recovery of the pigments concerned. Measurements of temporal absorption changes do not appear to have been made on passing from regions of chlorophyll a to b absorption, or from chlorophyll to carotenoid regions. These would be of interest, and conceivably rewarding.

Another, more trivial, explanation of the chromatic transients might be a temperature effect. Inactive absorption of certain wave lengths of light (e.g. by chlorophyll a in red algae) might slightly warm the tissue, giving rise to an increasing O_2 evolution (e.g. the slow rise in phase d, fig 5). Then on transfer to a more effective wave length (e.g. 540 or 560 mμ) the warmed tissue might show temporily higher photosynthesis (cusp, (a)) followed by depression (b) as the tissue cooled. Since the volume of liquid



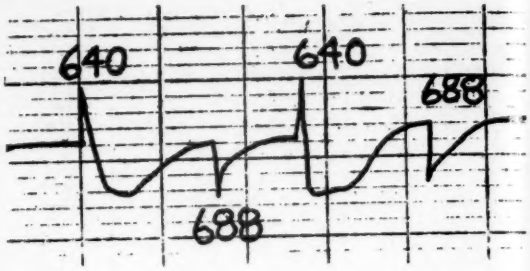


FIG. 3 (*left*). Recording of alternate exposures at 490 and 540 mμ. (a), above, low intensity; and (b), below, higher intensity (about 3 times that in a). Time marks, 7.5 minutes apart.

FIG. 4 (*right*). Recording of alternate exposures to 640 and 688 mμ of intensities such as to give equal steady state photosynthesis. Time marks, 7.5 minutes.

is large (and can be circulated without loss of the transients), the tissue thin (1 or 2 cells thick) and closely appressed to a good conductor (platinum), it is believed that such warming effects are negligible. Also, the effects in the carotenoid region of Ulva absorption (where efficiency is slightly low) resemble those in the phycobilin region of Porphyra (where efficiency is high). And there is no very great difference in the efficiencies of chlorophyll a and b, yet transients still occur in their absorption regions. So temperature changes are probably not involved.

The most likely explanation is that there are alterations of respiratory rate in different spectral regions, as found by Emerson (8) after illumination at 480 mμ, and offered by him as a suggestion to explain the chromatic transients. (See discussion in ref. (1)).

Upon this basis, the spike (a) might represent an initially higher rate of photosynthesis, occurring before respiration was secondarily promoted (e.g. by photooxidation, or the accumulation of photosynthetic intermediates). As such respiration developed the valley (b) would become evident. Meanwhile, however, the basic photosynthetic rate might also be slowly increasing, causing the recovery phase (c). If this is the case, such respiration would apparently be promoted more by chlorophyll b than by a (and by carotenoids or phycobilins more than by chlorophylls). On return to chlorophyll a absorption, this enhanced respiration would persist for a short time, giving the depression (d), which, however, passes away as the respiratory rate returns to normal (fig 5).

This is essentially the mechanism invoked by Brackett et al (3) to account for the time course of Chlorella photosynthesis, and may correspond to the carbon dioxide changes on illumination and darkening, reported by Decker (6, 7).

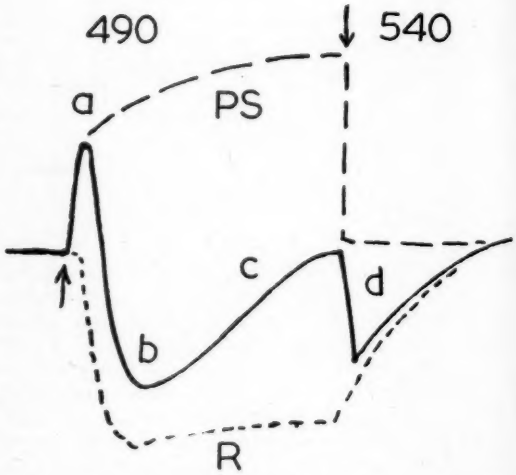


FIG. 5. Suggested explanation for the chromatic transients at 490 and 540 mμ. Photosynthesis (PS), initially at 540 mμ, is assumed to increase on illumination with the shorter wave length (1st arrow). The respiration (R) (shown as downward movement) also increases, after a slight lag, and then remains steady or perhaps decreases slightly. On return to the longer wave length, 540 mμ (2nd arrow), photosynthesis drops to original level and respiration slowly returns to normal. The solid line represents the observed time course of oxygen evolution, with the characteristic cusp (a), depression (b), recovery (c) and (at the longer wave length) depression (d) with recovery.

What basis, other than kinetic ingenuity, is there to support such respiratory changes during photosynthesis? The direct evidence from mass spectrometry is mostly negative (4), although there have been reports of respiratory enhancement in some species (5, 5 a, 10). But such measurements are not reported at isolated spectral regions. For these we are forced to depend upon the respiratory rate after illumination. Here Emerson and Lewis found increased respiration after absorption by carotenoid pigments (8); and at least some of our light-dark transients indicate this (e.g. fig 1). That it is not invariably found may be due to the time relations; evidently the enhanced respiration following chlorophyll b absorption is short lived, judging by the shape of phase (d), (compared to that after carotenoid absorption). It might not last much longer than the duration of the dark downward slope, hence be missed. That due to carotenoids (and phycobilins) is longer lived and often shows up as a post illumination depression.

The effect of temperature upon the transients (to be described elsewhere) seems to corroborate the role of respiration.

Whatever explanation we invoke for the chromatic transients, it is at least evident that the several pigments of the green algae (like those of the red) behave somewhat differently. They all sensitize photosynthesis, but on changing from absorption by 1 pigment to another—even as close together as 490 and 540 mμ—there are differences in kinetics which suggest that they may be doing different things, not merely at different rates.

Summary

Photosynthetic O_2 production by the marine green alga *Ulva lobata* was followed amperometrically by a polarized platinum electrode in direct contact with the tissue. Characteristic alterations in rate ("chromatic transients") were found when the tissue was alternately illuminated (without intervening dark periods) by light chiefly absorbed by a) carotenoids vs chlorophylls; and b) chlorophyll a vs chlorophyll b.

At 490 mμ (carotenoid absorption) there is a preliminary increase of rate (cusp) followed by a depression of 2 to 3 minutes, after which the rate increases and becomes steady. At 540 mμ (chlorophyll absorption) the rate drops about 10 %, then slowly recovers to normal. These transients may be observed repeatedly on each such alternation of wave length.

Transients similar to the carotenoid curves were found at 640 to 650 mμ (predominantly chlorophyll b absorption), while at 688 mμ (chlorophyll a) the transients resembled those at 540 mμ.

It is suggested that the transients are due to increased respiratory rate induced by carotenoid (or chlorophyll b) absorption. This agrees with the respiratory promotion observed by Emerson and Lewis after illumination at 480 mμ.

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INDUCTION OF FLUORESCENCE IN QUINONE POISONED CHLORELLA CELLS[1,2]

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The efficiency of fluorescence quenchers has been shown to be dependent on the concentration of the fluorescing dye. This effect was attributed to the occurrence of the migration of electronic excitation (6). In view of the special importance of this problem in the theory of the photochemical aspect of photosynthesis, it was found desirable to look at a possible difference between the efficiencies of fluorescence quenching of chlorophyll by quinone in vitro and in vivo. However, our attention was soon diverted toward a very striking change in the induction of fluorescence upon poisoning of Chlorella cells with quinone. Contrary to the impression given by one published observation (9), we found that quinone at moderate concentration did not suppress the induction of fluorescence completely. On the other hand under suitable conditions, the mean fluorescence level is depressed and a new induction course appears, the main feature of it being a rapid rise of fluorescence followed by a slower decay to a steady state.

The general significance of the Hill reaction and related processes is now well established: mechanical separation (isolated chloroplasts) or chemical inhibition (quinone reaction with Chlorella) uncouples the water-photolysis mechanism from the key hydrogen acceptor of the CO_2 reduction cycle. This must bring considerable simplification in the kinetic picture and might support the hope of untangling some of its components more easily. The present report will try to illustrate this point of view.

MATERIAL AND METHODS

Algae (*Chlorella pyrenoidosa*) were grown in Knopp solution at 23° C with 2 % CO_2-air mixture bubbling at the rate of 1.5 l per minute. Light was supplied by 2 banks of 3 fluorescent tubes (16-watt), 1 bank on each side of the culture flasks. For use, cells were centrifuged and resuspended in phosphate buffer 0.1 M, pH 6.4 plus KCl 0.05 M. A stock solution of purified quinone was added to the suspension giving a quinone concentration of 3×10^{-4} M (unless otherwise stated) and chlorophyll concentrations from 0.03 to 0.05 mg per ml. The mixture was incubated for ca. 10 minutes before the measurements were made.

The fluorescence was excited with monochromatic light at 670 mμ (half-band width: 13 mμ) from a Bausch and Lomb grating monochromator operated with a tungsten ribbon lamp. The fluorescent light was passed through a 2nd monochromator of the same type to the detector, an infra-red sensitive photomultiplier tube (Dumont, type K 1292). This 2nd monochromator was set at 725 mμ with a half-band of 26 mμ.

Fluorescence curves were automatically recorded with a galvanometer and a Photodyne recorder (Sefram); in several instances, oscillographic recording has been used. The cell suspension was introduced in a narrow vertical glass tube 1.5 mm inside diameter. A 10 mm section of this tube was illuminated with the exciting light which had a maximum incident intensity of ca. 3500 erg/sec cm² which will be referred to as I = 100. Constant temperature was maintained with a water jacket surrounding the glass tube. The fluorescence measurements were corrected for stray light using a correction factor estimated from measurements of the light scattered by light bleached cells.

RESULTS AND DISCUSSION

Figure 1 is a typical fluorescence curve, recorded under high exciting light intensity. Letters O, P and S have been used throughout this report as representing the initial, maximum and stationary levels of fluorescence; they divide the fluorescence curve into 2 main phases: O–P and P–S. We will also make extensive use of the quantities p_M, p and s, as defined on figure 1.

Several basic observations are to be noted first: 1.) Level S lies only slightly above level O, and conditions can be chosen that will maximize the ratio p_M/(light intensity): low temperature and high light intensity. 2.) The illumination can be interrupted for short periods of time during the P–S phase without significantly disturbing the time course of p. 3.) The time curve of p is best described by the equation:

$$1/p = A + Bt \qquad (1)$$

where A and B are constants and t is time. However, the validity of equation 1 is restricted to the end of the P–S phase, the 1st points lying definitely above the straight line (fig 2). 4.) The whole fluorescence curve can be repeated several times if the preparation is allowed to stay in darkness for a minimum period of time. The minimal interval depended on the temperature. Recording the fluorescence peaks at shorter periods of dark rest gives intermediate p_M values, which fall on a smooth curve when plotted as a function of dark time (fig 3). This process will be referred to as the recovery phase.

[1] Received March 23, 1959.

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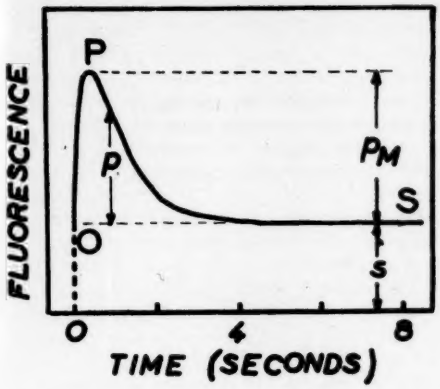
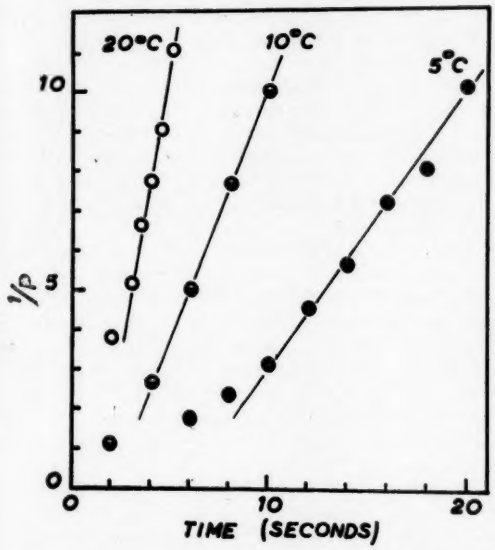
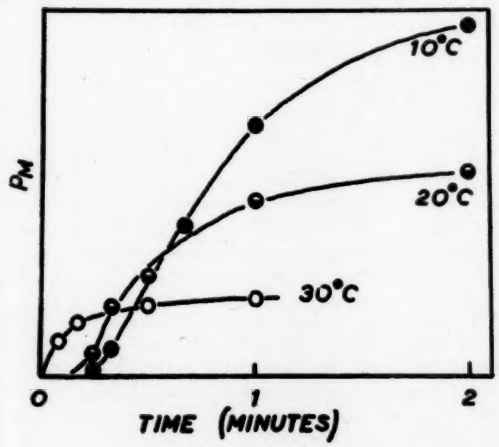
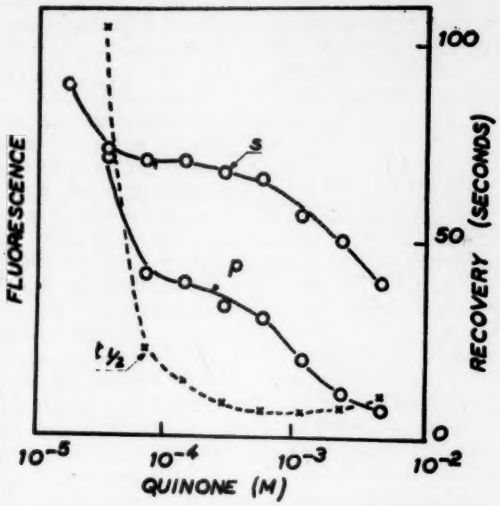








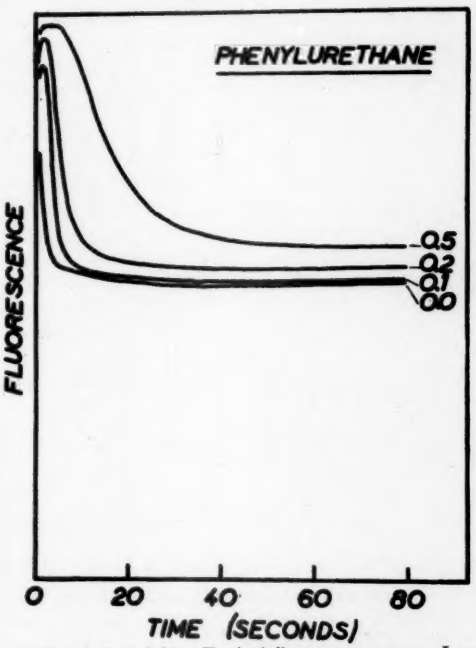


FIG. 1 (*top left*). Typical fluorescence curve. I = 100, 18° C (redrawn from oscillographic recording; symbols in text).

FIG. 2 (*center left*). Variation of 1/p during the P–S phase at different temperatures. I = 100.

FIG. 3 (*bottom left*). Recovery phase at different temperatures. I = 100. Note increase of final p_M value with decreasing temperature.

FIG. 4 (*top right*). p_M, s and half-time of recovery ($t_{1/2}$) as a function of quinone concentration. I = 100, 18° C.

FIG. 5 (*bottom right*). Effect of phenylurethane on fluorescence induction with quinone. I = 100, 18° C. Numbers are volumes in ml of aqueous saturated phenylurethane solution per ml of mixture.

The above mentioned observations will serve as the basis of our working hypothesis. One important, although somewhat independent assumption, is that the fluorescence emission can be split into 2 distinct parts: a constant back-ground of magnitude s, and a part subject to induction of variable magnitude p. The latter, which is all that concerns us here, is supposed to arise from a chlorophyll complex whose change in composition is partly reflected in the fluorescence change during the induction period. Specifically, 1 form of this complex, labelled ChlP, is fluorescent and non-photoactive: p is a measure of its relative concentration. Two other forms of the complex are needed to complete the picture: a non-fluorescent photoactive form, ChlO and a non-fluorescent non-photoactive form, ChlS. The 3 forms appear in the following sequence. Before illumination, the total amount of the complex is present in the state ChlO. When light is turned on, ChlO is photochemically transformed in ChlP: this accounts for the rapid fluorescence rise O–P. The next step P–S is essentially a thermal bimolecular process whereby ChlP combines with a substance X′ whose concentration at first lags behind, then equals that of ChlP near the end of the decay. This is in agreement with the 2nd observation and the asymptotic behavior of the P–S phase towards equation 1. This assumption in turn implies that, during the photochemical phase O–P, ChlO dissociates into ChlP plus a substance X which is then transformed in X′, an intermediate reaction that will at first delay the disappearance of ChlP by the bimolecular reaction. The end product of the latter reaction is the 3rd form of the complex, ChlS. A further reaction will transform ChlS in ChlO, thus closing the cycle. This is a slow process which, according to the 4th observation, gradually rebuilds ChlO during the recovery phase.

Summarizing, the chlorophyll complex undergoes the following sequence of reactions:

$$\text{O–P phase: ChlO} \xrightarrow{k^*I} \text{ChlP} + \text{X} \qquad (2\,a)$$

$$\text{P–S phase: X} \xrightarrow{k_1} \text{X}' \qquad (2\,b)$$

$$\text{ChlP} + \text{X}' \xrightarrow{k_2} \text{ChlS} \qquad (2\,c)$$

$$\text{Recovery phase: ChlS} \xrightarrow{k_3} \text{ChlO} \qquad (2\,d)$$

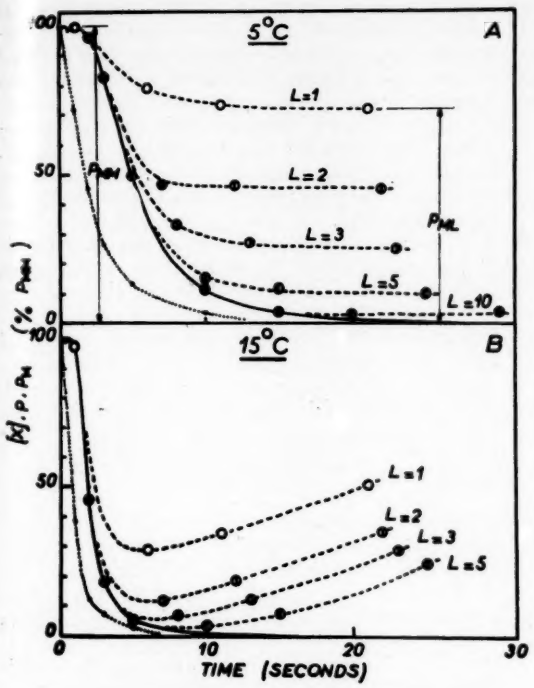
It is to be noted that points O, P and S in figure 1 correspond to the chlorophyll complex present in the state ChlO, ChlP and ChlS respectively. This, as well as attributing a specific reaction or group of reactions in scheme 2 to a given phase of the induction curve, is only legitimate in so far as the rate constants fall in the order: $k^*I \gg k_1$, $k_2 \gg k_3$. High light intensity, low temperature and medium quinone concentration are conditions typically fulfilling these requirements. Under different conditions (as will be seen below), one cannot draw such a sharp distinction between the successive parts of the induction curve.

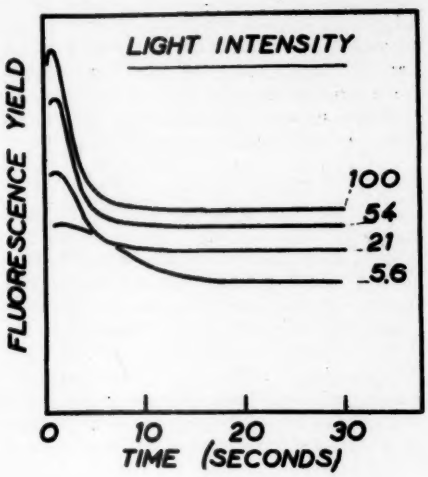
Quinone Concentration: The characteristic modifications of fluorescence induction here described make their appearance at quinone concentrations as low as 10^{-5} M. The action of quinone at such low concentrations is indicative of a primary inhibition of some important catalyst of the CO_2 pathway. On the other hand, we believe that true fluorescence quenching is not effective until the concentration is above 3×10^{-4} M (fig 4). According to Livingston and Ke (7), half-quenching concentrations in solution range from 8.1 to 9.6×10^{-3} M, depending on solvent. The rate of recovery, as measured by its half-time, is strongly dependant on quinone concentration (fig 4). Such a dependance can also be seen with Chlorella cells first exposed to the action of quinone, and then washed several times. The modification of fluorescence induction is not reversed—a fact reminiscent of the well known irreversible loss of photosynthetic ability—but the rate of recovery decreases with increasing number of washings. This points to attributing reaction 2 d to the reduction of quinone; if so, ChlS would be the reduced form of the complex.

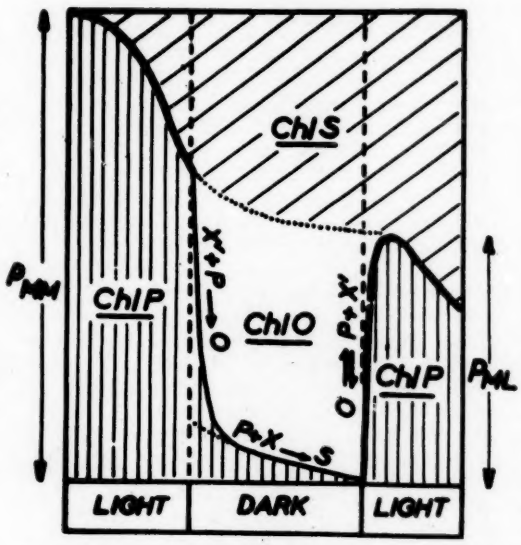
Inhibitors: Among inhibitors known to affect the Hill reaction, we have found that phenylurethane and *o*-phenanthroline had similar strong effects, whereas the only action of hydroxylamine was the lengthening of the recovery phase. Upon adding increasing amounts of one of the active inhibitors at room temperature, it is found that p_M increases up to a maximum value and that the P–S phase starts with a plateau and gets progressively slower (fig 5). These results can be understood on the basis of a specific inhibition of reaction 2 b. In particular, a maximum value of p_M means that the balance between production and consumption of ChlP, which determines the concentration of this substance at point P, is shifted toward maximum accumulation. Low temperature (see below) obviously must have similar effect.

Another effect of phenylurethane which would require a special explanation is the increase in the rate of recovery. As this accelerates the cycle, the stationary level S tends to rise slightly, as can be seen on figure 5.

P–S Phase in Darkness: Scheme 2 suggests that, providing the recovery reaction 2 d can be kept slow enough and sufficient light has been given to terminate the photochemical reaction 2 a, the P–S phase should not depend on sustained illumination and should proceed to completion in darkness (not only for short periods of time, as specified in the 2nd observation). Accordingly, the preparation was pre-illuminated during a time L and the peak of fluorescence p_M was recorded after a dark period D. Figure 6 is a plot of p_M as a function of L + D for several durations of pre-illumination at 2 temperatures. The P–S curve in continuous illumination is also given for comparison. It is seen that the dark evolution approximates the light one for only short periods of time following the end of pre-illumination and that there is accumulated a limited amount of ChlS—as measured by the difference $p_{M\infty} - p_{ML}$ (see fig 6 A)—







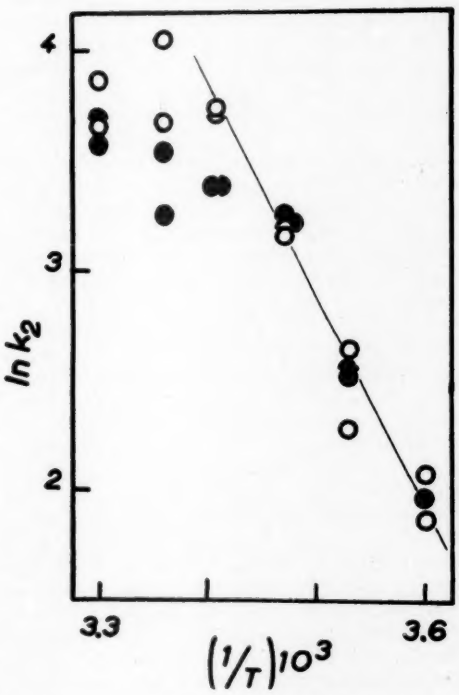


FIG. 6 (*top left*). P–S phase in darkness at two temperatures. (———) P–S in continuous illumination, (- - - -) p_M curve following pre-illumination L (final p_M value at 5° C is p_{ML}), (. . . . .) time curve of X.

FIG. 7 (*bottom left*). A sketch of the kinetic evolution of the system upon interrupting light during the P–S phase. Heavy inked line corresponds to the fluorescence curve.

FIG. 8 (*top right*). Effect of light intensity on fluorescence induction with quinone. 5° C.

FIG. 9 (*botom right*). k_2 (arbitrary unit) as a function of absolute temperature T in the range 5 to 30° C. Cells grown in normal (O) and 70 % normal light (●).

which increases with L up to the maximum possible value. At higher temperature (fig 6 B) reaction 2 d proceeds at an appreciable rate and prevents the accumulation of ChlS from reaching a stable level, as the ascending curves of p_M show. The more likely explanation of this dependance is that reaction 2 a is in fact a photochemical equilibrium:

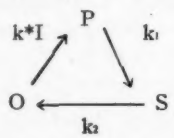
$$\text{ChlO} \xrightarrow[k']{k^*I} \text{ChlP} + X \qquad (2a')$$

As long as equilibrium 2 a′ is shifted far to the right by high light intensity (pre-illumination), X disappears by reaction 2 b, as was assumed above. As soon as light is turned off, equilibrium 2 a′ quickly shifts to the left, thus returning part of ChlP to ChlO and bringing reaction 2 b to a standstill. The remainder of ChlP keeps following the "normal" path 2 c to the extent of the amount of X′ produced during the preceding light period. When light is turned on again, the maximum amount of ChlP compatible with the instantaneous state of the kinetic system is quickly formed through equilibrium 2 a′ and appears as p_{ML}. This complex behavior is schematically depicted on figure 7. Thus, the maximum amount of ChlS produced following pre-illumination L can be used as a measure of the amount of X consumed during time L; the time course of X consumption by reaction 2 b has also been plotted on figure 6.

Light Intensity: The effect of this variable is illustrated by figure 8; time curves of relative fluorescence yield have been used, enabling direct estimation and comparison of the amount of ChlP present under different light conditions. The increase of ChlP concentration with light intensity at point P is self explanatory. The lengthening of the P–S phase when light intensity decreases results from the above given considerations: at low light intensity, equilibrium 2 a′ lies in the middle of its maximum excursion, the more to the left the lower the intensity. This keeps the concentrations of ChlP and X low, thus slowing down the subsequent dark reactions. That the S level rises when light intensity increases is evidence that reaction 2 d is not so slow in this instance as to prevent the cycle from running at a steady rate; hence there is still a small amount of ChlP when the stationary level S obtains. Similarly, at low light intensity, ChlO is likely to become a non-negligible form of accumulation of the complex. This tends to depress the concentration of ChlP. It must be noted that in such a case the S level lies appreciably above the O level and that, since for practical reasons ChlP concentrations are measured with reference to the S level, equation 1 should not be strictly valid. Physiological factors affecting the difference between the 2 levels have not yet been investigated. However, we infer from the general validity of equation 1 that the difference is usually small.

Temperature: The effects of this variable on the thermal processes included in scheme 2 bear some similarity with those described under the section Inhibitors and do not need special explanations. Lowering the temperature increases p_M, depresses the rate of the P–S evolution and of the recovery reaction (see fig 6 and 3). The activation energy of reaction 2 c can be computed from the variation of k_2 (equation 1, where $B = k_3$) with temperature (see fig 2). Figure 9 is the corresponding Arrhenius plot. A deviation from the linear relation can be noticed at high temperature. We have already emphasized that exact correspondence between each step of the cycle and successive phases of the induction curve was not to be expected with every set of experimental conditions. At high temperature, each step tends to affect the following ones more or less and the rate of fluorescence change can no longer be a function of a single rate constant (e.g., k_2 for the end of P–S phase). It can be seen that the sign of the discrepancy is what the above analysis would predict. From the straight part of the curve in figure 9 an activation energy of ca. 20 kcal can be computed.

Concluding Remarks: It seems premature to draw conclusions from a comparison between our results and what is known of the induction phenomena of fluorescence in normal algae. We have still to investigate the significance of variations in parameters of the induction curves (for example, absolute and relative variations of p_M and s with culturing conditions, especially light); also, keeping in mind that any photochemical cycle has to operate a light-sensitized hydrogen transfer, we have to locate the point at which O_2 evolution, water re-loading and hydrogen unloading to the acceptor will occur. However, we believe that a discussion of the basic kinetic concepts here postulated, as compared with other theoretical approaches, might be of some relevance for the general problem of induction. Rabinowitch (10) has given a thorough analysis of the two possible mechanisms of induction, namely the building up of intermediates and the activation of catalysts. He also pointed out the weight of experimental evidences in favor of the second mechanism, specifically as postulated in the Gaffron-Franck theory (3, 5). One of the points that Rabinowitch made was that the first mechanism could not explain the characteristic waves of induction, giving only a gradual approach to the steady state. In fact, it is possible to find kinetic systems that will respond with transient waves to a sudden change of condition. It can be demonstrated that even a simple cycle such as:



—a simplified version of our cycle—will yield monotonous induction curves as well as one or several maxima before the establishment of a steady state, depending on the choice of a suitable set of reaction-

rate constants. A salient postulate in the Gaffron-Franck theory is that the O_2-liberating catalyst C is de-activated in the dark and becomes autocatalytically re-activated in light; this triggers a mechanism of photoperoxide accumulation and narcotic production which slowly subsides as catalyst C starts to operate. Using a very fast amperometric technique (time resolution of the order of 0.5 sec) for the O_2 determination, Joliot (unpublished) has found that, after not too long dark periods, the O_2 evolution begins with a burst followed by a characteristic induction period. The maximum rate at the start of the O_2 burst equals the steady photosynthetic rate, when the experiment is carried out at low light intensity. This shows that inhibition of the O_2-liberating catalyst (which Joliot also observed) need not be considered as the only source of induction.

As pointed out by Rabinowitch, one difficult problem in connection with the Gaffron-Franck theory is to understand how a small number of narcotic molecules produced at the beginning of illumination can affect the fluorescence of the totality of chlorophyll molecules. This question obviously cannot be asked as such in our theory, but it has a very interesting counterpart: how is the photochemical reaction 2 a to proceed so rapidly? Indeed, we have compared (observations to be published) the experimental rate constant of the fluorescence initial rise O–P, which we attribute to the photochemical transformation of the photoactive form ChlO, with a calculated rate constant assuming that reaction 2 a proceeds with unit quantum yield and that there is negligible mutual shading between the chlorophyll molecules; this "optimum" calculated value depends on the light intensity and the absorption coefficient, which we took to be of the same magnitude as in chlorophyll solutions. We found that the measured rate constant is from 100 to 200 times larger than the calculated one. In other words, this would mean that the absorption coefficient of ChlO stands in this ratio with the absorption coefficient of chlorophyll in solution. This is very unlikely. The alternative to explain how the ChlO molecules receive more energy than permitted by their own photic "cross section" is that an average number of 100 to 200 chlorophyll molecules in a special state, distinct from the complex state, supply their absorbed energy to a single molecule of this complex in its ChlO form. This would not only be in agreement with the Emerson-Arnold concept of "photosynthetic unit" (2), but would also be in favor of one of its possible interpretations, namely the electronic excitation migration to the "reaction center" (4), as opposed to the material diffusion of the photochemical products or even of the center itself. To us, this would also provide a strong support for the assumption we made as to the heterogeneity of the fluorescence emission. The constant background would arise from the bulk of the chlorophyll acting as photic acceptor and the variable fluorescence from the few molecules of chlorophyll complex in charge of the photochemical transformation of the transferred energy. A similar hypothesis as to the state of chlorophyll in vivo has been repeatedly made since the original elaboration of Seybold and Egle (8). Recently, Franck (1) has proposed a very detailed model—based on the same concept of heterogeneity —in which the 2 functions of chlorophyll (light absorption and photochemical transformation) are likewise attributed to distinct fractions of the pigment.

Summary

The kinetics of the induction phenomena of fluorescence in quinone-poisoned Chlorella cells has been studied. The observations are consistant with the following assumptions.

1) Part of the chlorophyll is involved in a photochemical cycle as a complex that can assume 3 different forms.

2) Successively, there is a non-fluorescent photoactive form, a fluorescent non-photoactive form and a non-fluorescent non-photoactive form.

3) This photochemically participating fraction of chlorophyll is responsible for the variable part of the fluorescence emission, whereas the constant fluorescence background arises from a different fraction which only acts as a photic acceptor.

The author gratefully acknowledges the facilities kindly extended by Professor R. Wurmser in the Laboratoire de Biologie physico-chimique and his continued interest while this work was in progress. The author is also indebted to P. Joliot for numerous discussions which greatly helped clarify the subject.

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QUANTUM REQUIREMENT FOR PHOSPHOPYRIDINE NUCLEOTIDE REDUCTION IN PHOTOSYNTHESIS [1, 2]

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Experiments will be reported concerning the number of absorbed light quanta needed to reduce 2 molecules of pyridine nucleotide (PN) in photosynthetic cells and spinach chloroplasts. We will call this number the quantum requirement of PN reduction. If CO_2 is reduced via reduced pyridine nucleotide (PNH), then 2 molecules of PNH are needed to reduce 1 CO_2 molecule. If the quantum requirement of PN reduction is large, compared to the quantum requirement of photosynthesis (defined as the number of absorbed quanta needed to reduce 1 CO_2 molecule), then this would indicate that the reduction of CO_2 does not mainly occur via PN; on the other hand if the number is of the same order of magnitude, then this would support the hypothesis that PN is an intermediate in the reduction of CO_2.

The reduction of PN was studied in intact photosynthesizing cells by making use of the long known fact that reduced di- or tri- phosphopyridine nucleotide (DPNH or TPNH) fluoresces in the blue region, when excited by near-ultra-violet radiation, while oxidized pyridine nucleotide does not. Photosynthetic cells, when excited by the mercury line at 366 mμ, show a blue fluorescence, which appears to increase upon illumination with red light. The red light causes photosynthesis to proceed at a much higher rate than the weak fluorescence exciting radiation, but does not cause blue fluorescence. Spectrofluorometric studies showed that the fluorescence increase was caused by PN reduction (5, 11). It was not established whether DPN or TPN was reduced. The observation that the steady state PNH concentration during illumination in Chromatium (the only species that was studied in this respect) was lower at higher concentration of CO_2 in the medium, suggested that PNH participated in the reduction of CO_2 (10).

Also results of other authors are consistent with this suggestion. Various workers demonstrated reduction of DPN or TPN by cell-free preparations from photosynthesizing organisms; recently even appreciable accumulation of PNH was reported (3, 8, 12).

Although it has been demonstrated that PN is reduced by illimunated extracts, that enzymes are present which are able to catalyze the reduction of CO_2 (at a slow rate) by TPNH in the presence of ATP (cf. 1, 16), and (by means of the fluorescence experiments), that PN is also reduced upon illumination of intact cells, it has not yet been proven that PNH participates in the reduction of CO_2. As will be shown, results of the experiments on the quantum requirement of PNH reduction in intact cells and chloroplast suspensions strongly support this hypothesis.

METHODS AND RESULTS

The rate of the reduction of added TPN by illuminated spinach chloroplasts was determined from a recording of the absorptance at 350 mμ. The absorptance (ratio of absorbed and incident light flux) was measured with a somewhat modified Zeiss spectrophotometer connected to a Varian recorder (2). The modified part of the apparatus is shown in figure 1.

The suspension was in a quartz vessel V of 1 mm thickness, placed in the vessel carrier of the spectrophotometer. The measuring beam from the monochromator, which is set at 350 mμ, passes a filter F_2, which transmits a narrow band around 350 mμ, but stops the red actinic light from a projector. Corrections for scattering of the suspension were made small by placing an opal glass plate O close to V (cf. 14) and close to a blank vessel, which was also placed in the vessel carrier. The correctness of the absorptance measurements was checked by adding small amounts of DNPH of known absorbancy.

The number of actinic quanta absorbed is equal to the number of incident quanta, multiplied by the absorptance of the actinic light. The number of incident quanta per sec per cm^2, was measured by moving a small calibrated silicon cell (Lange) into the actinic beam.

The absorbancy of the suspension for the actinic light was measured, after removing filter F_2, and setting the monochromator at wave length 668 mμ of

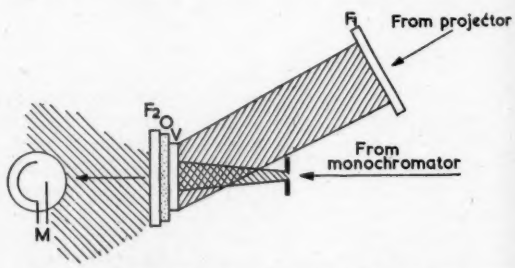

FIG. 1. Schematic diagram of part of the apparatus for measuring the rate of PN reduction by a chloroplast suspension, which is illuminated by filtered light from a projector.

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the actinic light. The apparent rather small absorbancy at 720 mμ, which is probably due to scattering, since chlorophyll does not absorb at this wave length, was subtracted from the measured absorbancy at 668 mμ, and the result was taken as the "true absorbancy" of the suspension. Since the true absorptance was close to 90 %, serious errors appear unlikely in this measurement.

The quantum requirements were determined for intensities of actinic light, at which the rate of TPN reduction increased approximately linearly with light intensity. Also DPN was reduced, but at a lower rate than TPN, especially at the higher light intensities. The data indicated (2) that, at very low light intensities, the rates and thus the quantum requirements of DPN and TPN reduction approached each other, and also that TPN and TPNH strongly inhibited DPN reduction.

The following quantum requirements for TPN reduction were found with 5 different chloroplast preparations from commercially obtained spinach: 12.8 11.6 11.6 11.0 14.0.

Application of the absorption method for measuring PN reduction in vitro is difficult, at least in normally colored photosynthesizing organisms, because of the smallness of the effect, and because of the uncertainty, caused by absorption changes by other cellular components, such as cytochromes. The increase of fluorescence in the blue region upon illumination of suspensions of photosynthesizing cells was found to be a more unique and sensitive indication of PN reduction (5), and was taken as a measure of the number of PN molecules reduced. The blue fluorescence was calibrated by measuring the increase in fluorescence, caused by adding a known amount of DPNH to the suspension. If we assume that a molecule PNH formed inside the living cell has the same fluorescence yield as a molecule DPNH outside, then the number of reduced PN molecules can readily be calculated. However, this assumption is probably not correct, since the shift of the maximum of the fluorescence difference spectrum in vivo from about 460 to 440 mμ (5) indicates that an appreciable part of the PNH molecules formed in the light must be bound to cell constituents. Binding of DPNH to alcoholdehydrogenase was found to cause not only a shift in the maximum from 460 to 440 mμ, but also a pronounced increase in fluorescence (6, 15). This phenomenon has been observed for binding of PNH to various other enzymes (cf. 15, 19). If all PNH molecules in vivo possess an equally high fluorescence yield as those bound to yeast alcoholdehydrogenase (6), then the number of molecules PNH calculated on the above mentioned assumption has to be divided by a factor of about 4. We do however not yet know the fraction of PNH, which is bound in vivo, nor its precise fluorescence yield. The increase in fluorescence yield of PNH bound to various enzymes does not appear to be higher than 4. We therefore have multiplied the originally calculated numbers by a factor between 1 and one fourth. For chloroplasts, which are able to produce TPNH from added TPN in an amount, which is in excess of what can be bound by cell constituents, the uncertainty is smaller.

The actinic light used was a narrow band in the red or, for Chromatium, in the near infra-red region. The incident intensity in ergs (and thus in quanta) per cm^2 per sec was determined by means of a calibrated thermopile. The vessel containing the suspension was the same as that described above and its absorptance was measured in the same way.

Upon onset of photosynthetically active actinic light, the PNH level increases steadily during the 1st second or seconds. The initial rate of increase is higher at higher intensities of actinic light, but approaches a maximum.

The quantum requirement was determined for the initial rate, at an actinic intensity below that, needed for saturation of the initial rate. The quantum requirements for PNH reduction were between the following limits (cf. 2). For Chlorella: 8.6 and 34 : for the purple bacterium Chromatium: 5.6 and 22.4 : for suspensions of spinach chloroplasts to which TPN had been added: 8.6 and 18.

The spread in these values is due, as we have explained above, to the uncertainty in the fluorescence yield of PNH in the cells, as compared to that of DPNH in water solution.

Discussion

The quantum requirement for TPN reduction found for spinach chloroplasts with the absorption method, is not inconsistent with that found with the fluorescence method for different species, although the latter values have a great uncertainty. The quantum requirement for spinach chloroplasts was found to be about 12, as compared to 32, observed by other authors (13). The explanation for this large discrepancy is not clear. It is possible that in the latter investigation the rate of TPN reduction decreased during the 10-minute period of illumination, after which the TPNH formed was determined. In our calculations, the initial rate was used, since the rate decreased in time, perhaps because of TPNH formation. Another possibility might be that the kind of spinach used or the preparation of chloroplasts may have been the source of the discrepancy. The quantum requirements of 12 is not very different from the quantum requirements for CO_2 reduction by intact organisms as found by many other investigators, in the first place by Emerson and co-workers (cf. 7).

Most quantum requirements for CO_2 reduction were between 7 and 12, and according to a critical review (7), the "true value" may be between 7 and 9. The lowest quantum requirement reported in literature was about 3 (17). It may however be argued that a quantum requirement lower than about 4 contradicts the second law of thermodynamics. The argument is briefly as follows (4). If the absorbed light energy were completely converted into chemical energy then about 3 quanta would suffice to reduce 1 molecule of CO_2 to the level of carbohydrate and to "oxidize"

2 molecules of water to O_2. However, light energy like heat, is not, as is often believed, a form of freely transformable energy. The maximum efficiency with which light energy can be converted into "high grade energy" depends, amongst others upon the "structure" of the light, in an analogous way as the maximum efficiency with which heat can be transformed depends, amongst others, upon its "structure." For instance the maximum efficiency, E, with which temperature radiation emitted by a source of T_1 degrees Kelvin, can be used to produce chemical or other high grade energy in a reaction vessel, kept at T_0 degrees Kelvin, according to a consequence of the second law of thermodynamics, equals $E = (T_1 - T_0)/T_1$, which is a number smaller than 1. If $T_1 = 1100$ and $T_0 = 300$ °K (room temperature), then $E = 0.73$. When the reaction vessel is placed inside a "black" body of this temperature, and the light energy passes through a spherical filter, which surrounds the vessel and transmits a band between 660 and 680 $m\mu$, and reflects all other light, then the intensity incident on the vessel is 0.17 mW/cm². This light cannot be converted into high grade energy with an efficiency better than 73 %, whether the vessel contains a photosynthesizing suspension or a photoelectric cell. Since all investigators seem to agree that the quantum requirement of a suspension of photosynthesizing algal cells in this light (possibly aided by "catalytic" amounts of light of shorter wave length) is not higher than for any other light regime, it follows that the quantum requirement for algae and higher plants must be equal to or higher than 3/0,73 or about 4. The same considerations can be applied to all "partial" reactions of photosynthesis, following light absorption, and may be helpful, when analyzing these reactions.

The quantum requirements determined by us, are consistent with the hypothesis that the reduction of CO_2 proceeds via PNH. The most precisely determinded one for TPN reduction in spinach chloroplasts is somewhat higher than those generally found for algae and for CO_2 reduction in Chlorella, but this may be due to the difference between unicellular algae and higher plants. The quantum requirement of TPNH reduction for living cells must be determined more precisely, and be compared with simultaneously determined quantum requirements for CO_2 reduction, before it can be stated with more certainty whether the quantum requirement of PN reduction is equal to or different from that of CO_2 reduction. The present data, however, indicate that at least an appreciable part of the CO_2 reduction occurs via PN. If this is true, then our data are consistent with the statement that the minimum quantum requirement of CO_2 reduction is appreciably higher than 4.

The quantum requirement for PN reduction in the purple bacterium Chromatium 5.6 to 22.4 may be compared to that for CO_2 reduction 8.5 to 13 (18), and that for the oxidation of cytochrome c 553, which appears to be about 8 (within rather wide limits of uncertainty) per 4 cytochrome molecules oxidized (9). These values suggest that, in purple bacteria, the reduction of CO_2 is mediated by PN, and, as Olson (9) concluded, the oxidation of substrate is mediated by a cytochrome.

Summary

By means of absorption spectrophotometry the number of light quanta, absorbed by a suspension of spinach chloroplasts, required to reduce 2 triphosphopyridine nucleotide molecules, was found to be about 12. The analogous quantum requirements for phosphopyridine nucleotide (PN) reduction by living cells, as determined by fluorescence spectroscopy from the initial rate of PN reduction upon illumination, were found for intact cells of the purple bacterium Chromatium and for Chlorella, to be between 5.6 and 22.4 and between 8.6 and 34 respectively. These quantum requirements are of the same order of magnitude as those found by various authors for CO_2 reduction, which indicates that phosphopyridine nucleotide participates in the reduction of CO_2.

It was argued that a quantum requirement for CO_2 reduction by algae of less than 4 contradicts the second law of thermodynamics.

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PRIMARY PHOTOCHEMICAL AND PHOTOPHYSICAL PROCESSES IN PHOTOSYNTHESIS[1]

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I. Excitons and Their Migration

In photochemical reactions in condensed systems, intermediary steps can occur between light absorption and the primary photochemical reaction: the photophysical processes of energy or charge transfer. Either the excited electron, or the hole it leaves in the absorbing molecule, may move, to be found at the end of the excitation period in a site far removed from the original locus of excitation. If the electron and the hole travel separately, the final effect is separation of charges—which can be also described as internal oxidation-reduction.

The electron-hole pair is often referred to as an exciton. When the excited electron and the hole are associated in a single molecule, the (perhaps superfluous) term "intramolecular exciton" is used. Figure 1 illustrates the different modes of transfer of intramolecular and intermolecular excitons (1).

The binding energy of the exciton may be so small that it can dissociate under the influence of thermal motion, or under the pull of an applied electric field (photoconductivity). If impurities are present in the system, or adsorbed on the surface, they may trap the electron, or the hole, or both.

Recently, it has been pointed out that the concentration of pigment molecules in the photosynthetic organelles is so high as to justify approaching photosynthesis from the point of view of phenomena in the solid state, making the above considerations relevant.

II. Structure of Chloroplasts

All chloroplasts contain lamellae—flat or involuted, thin extended layers, about 20 mμ thick. They are found also in chloroplast-free cells of the blue-green algae. The lamellae either fill out the whole body of the chloroplast (lamellar chloroplasts), or form cylindrical grana (granular chloroplasts). Figures 2 and 3 show both types of chloroplasts in the same plant. Figure 3 shows that in granular chloroplasts, the lamellar structure extends into the intergranular stroma; the grana are merely approximately cylindrical volumes in which the lamellae are denser and more numerous.

The high optical density of the lamellae in preparations fixed with osmic acid (as in figs 2 and 3) is due to higher concentration of precipitated osmium; and this in turn seems to be due to the presence of lipoids. Consequently, the lamellae must contain the greatest concentration of lipoids, while the stroma is more predominantly proteinaceous. In granular chloroplasts, absorption and fluorescence microscopy confirm that the (lipophilic) pigments are located in the grana.

Chlorophyll, whose molecule consists of a flat, slightly polar, colored conjugated ring system (chlorin) and a long, colorless tail (phytol), should accumulate on interfaces between the hydrophilic and the hydrophobic layers. Thomas and co-workers (2) compared the number of chlorophyll molecules in the chloroplasts of 8 species with the total surface areas of their lamellae. The results varied between 1.8 mμ^2 and 3.8 mμ^2 per chlorophyll molecule, while the amount of chlorophyll per chloroplast varied by a factor of 5×10^3.

Artificial chlorophyll surface layers (3, 4) exist in two forms: crystalline and amorphous. In the first form, the area requirement is about 0.45 mμ^2 (suggesting a 2-molecular layer), and the red absorption

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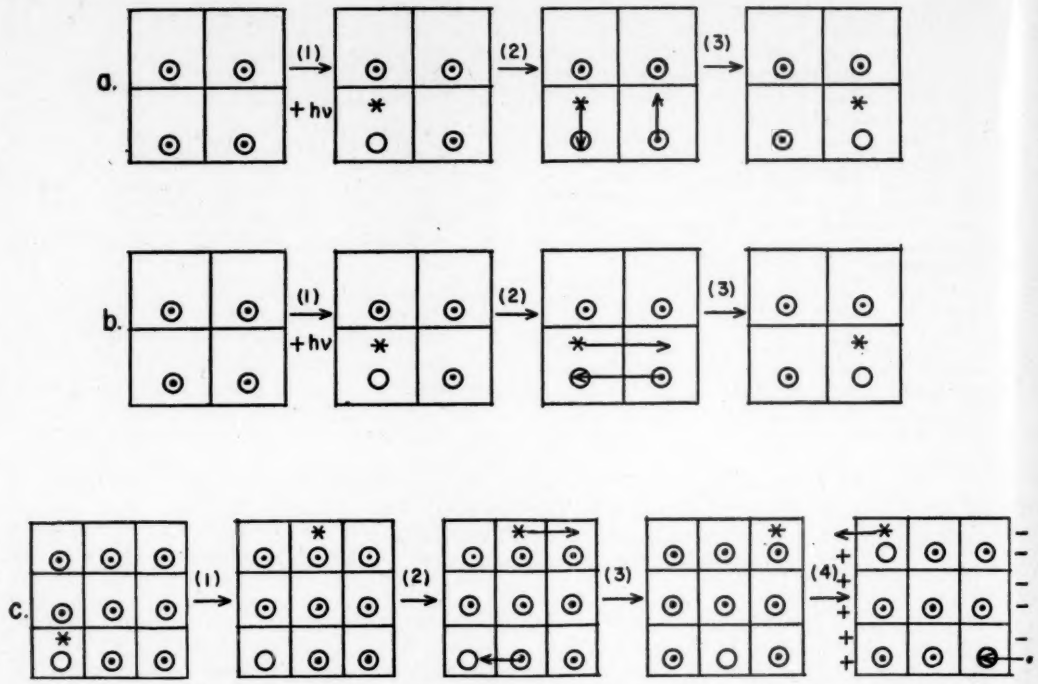

FIG. 1. Transfer of intramolecular and intermolecular excitons. ○, hole; ●, electron; and *, excited electron. Top row, creation (1) and transfer (2), (3) of intramolecular exciton—Förster-Heller-Marcus mechanism. Center row, same—Wannier mechanism. Bottom row, conversion of intra- into intermolecular exciton (1); its transfer (2), (3); and dissociation (4).

band lies at 735 mμ; in the second form, the area requirement is 1.06 mμ², and the red absorption band lies at 678 mμ.

In both types of artificial surface layers, the chlorophyll ring system must be stacked obliquely, since the actual area of this system—which would be required for the molecules to lie flat on the interface—is about 2.4 mμ². This is close to the average found by Thomas et al for chloroplasts. The position of the absorption band in vivo—at 675 to 680 mμ (fig 4)—shows the absence of crystalline, and supports the possible presence of amorphous monolayers.

The electron micrographs suggest the existence of flat chambers enclosed between pairs of lamellae in grana (fig 3). One can suggest that one product of the photochemical reaction (e.g., the reduction intermediate) is trapped in the chamber, while the other —the O_2 precursor—escapes on the outside. Lamellar chloroplasts, containing no such chambers, also are capable of photosynthesis; but some evidence suggests that their lamellae tend to associate in pairs; so that a similar separation mechanism could also operate in them.

The arrangement of chlorophyll in monomolecular layers was confirmed by Goedheer's (5) study of the (relatively weak) dichroism and the (pronounced) birefringence dispersion of large chloroplasts. According to Goedheer, his observations suggest that chloroplasts contain very thin (0.2 to 0.6 mμ thick) pigment layers, which are obviously monomolecular. The axes of the chlorophyll molecules seem to be only very imperfectly oriented, thus accounting for the weakness of the dichroism. Goedheer suggested that this may be due to the monolayer covering not a flat, but a grained surface. Spherical macromolecules can be, in fact, seen on the surface of lamellae (6). They have a diameter of 7 to 10 mμ, and thus a surface of 150 to 300 mμ² (fig 5). This should be enough to accommodate 150 to 300 adsorbed chlorophyll molecules, if the sphere could be covered uniformly—which is somewhat difficult if the macromolecules are incorporated in lamellae.

The hypothetical picture of protein macromolecules carrying 200 or 300 molecules is similar to the empirical picture of phycobilins as protein macromolecules (mol. weight ≅ 280.000) carrying 50 to 100 chromophores (S. and M. Brody (7)). The main difference may be in the additional association of the chlorophylls with lipoids (probably through the hydrophobic phytol tail), which prevents the extraction of chlorophyll macromolecules with water.

III. States of Chlorophyll in the Chloroplasts

Various evidence indicates that chlorophyll in the chloroplasts is present in at least two forms (see e.g., Krasnovsky et al (8) and Smith et al (9)). It has been suggested that one is monomeric and another aggregated. Apparently discordant measurements of life-time and of the quantum yield of fluorescence (in Chlorella) by Brody (10) and Latimer (11) can be reconciled by the assumption that about one fourth of the chlorophyll is in the fluorescent state (with a yield of approximately 10 %), while three fourths is non-fluorescent (giving an average yield of 3 %). Other observations (Brody (12)) make it appear likely that one non-fluorescent form of chlorophyll in vivo has an absorption band near 705 mμ, and a fluorescence band at 720 mμ (which appears only at low temperatures).

The monomeric and polymeric, fluorescent and non-fluorescent forms of chlorophyll in vivo probably mean different states in a monolayer—possibly distinguished by the density of the monolayer, or the kind of molecules with which it is associated. Franck (13) concluded, from kinetic considerations, that two types of chlorophyll molecules in vivo are characterized by contact with water, or with non-polar molecules, respectively. The former are fluorescent; no free electrons can occur in this hydrated part of the monolayer. The lipoid-protected part could be non-fluorescent, and could support the existence of free electrons. According to Platt (14), the non-fluorescent $n\pi$ excited state must be lower than the fluorescent $\pi\pi$ state in lipoid-bound, and higher than the latter in the hydrated form, thus accounting for the latter's capacity for fluorescence (fig 6).

Franck suggested that for the primary photochemical process in photosynthesis, 2 excited molecules are needed, one of which must be in the $\pi\pi$ excited state, while the other can be in the $n\pi$ state.

IV. Energy and Charge Transfer in Chloroplasts

The problem of energy transfer in photosynthesis arose in 1936, when Gaffron and Wohl (15) interpreted the results of Emerson and Arnold's flashing light experiments (showing that the maximum yield of O_2 per brief flash (10^{-5} sec) is about 1 molecule of O_2 per 2000 molecules of chlorophyll), by the assumption that about 2000 chlorophyll molecules act as a photosynthetic unit, an energy catch-basin associated with a single reduction site, in which the photochemical process takes place. The number 2000 must be reduced (perhaps, by a factor of 8) because the liberation of 1 molecule of O_2 requires several (perhaps 8) elementary photochemical processes. The photosynthetic unit is therefore most likely to consist of about 250 chlorophyll molecules. This makes it possible to consider identifying the unit with the pigment-bearing macromolecules in the lamellae, which according to section II, may carry 100 to 300 chlorophyll molecules.

Gaffron and Wohl said that cooperation between the many light-absorbing chlorophyll molecules and a single reaction center in a unit can be attributed to the migration, either of energy-rich particles generated at each chlorophyll molecule, or of energy quanta. The second picture has fascinated workers in photosynthesis in the last 20 years, without a definite answer being found as to its relevance.

Goedheer (5) observed a very low fluorescence polarization of phycobilin, suggesting that the excitation energy moves among the differently oriented chromophores attached to the macromolecule. In analogy, one could expect energy exchange also between chlorophyll molecules attached to a common macromolecule. It may perhaps take in several adjacent macromolecules, thus accounting for the rela-

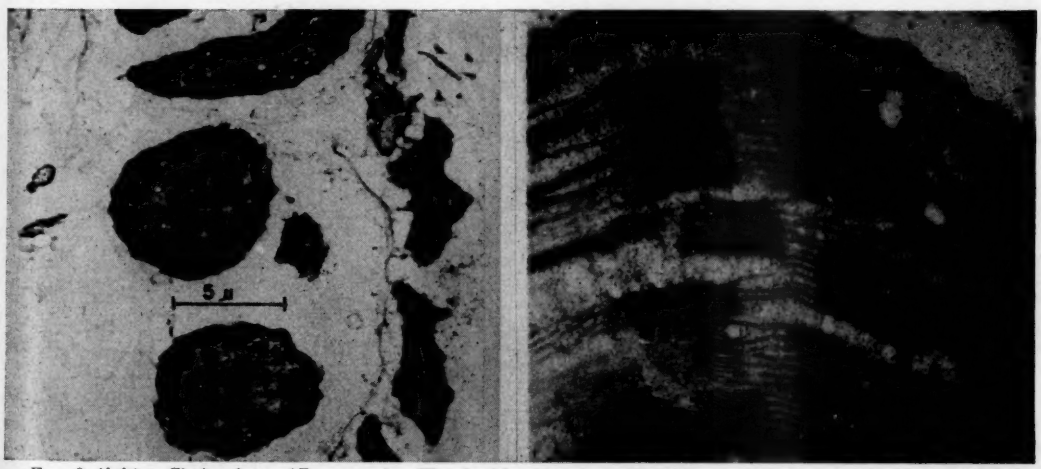

Fig. 2 (*left*). Cholorplasts (*Zea mays*). The 3 chloroplasts on the left are granular, the 2 on the right are lamellar. (After Vatter.)

Fig. 3 (*right*). Chloroplast structure (*Zea mays*). Cross section of a granular chloroplast, showing intergranular lamellae. (After Vatter.)

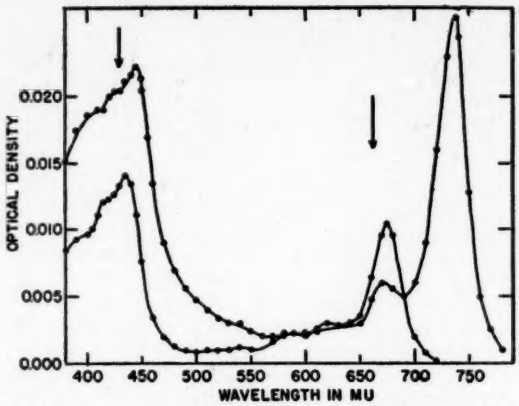

FIG. 4. Absorption curves of 2 types of chlorophyll surface layers on water (arrow indicates the position of the bands in solution). (After Jacobs et al.)

tively large number (at least 250) molecules in the unit; however, in this case, only one out of several macromolecules could carry an enzymatic reaction center.

Plant cells need a high concentration of pigment to provide sufficient light absorption; but they do not have enough space to provide a separate enzymatic conveyor belt for each chlorophyll molecule. A high ratio (chlorophyll) : (enzyme) is possible because even in direct sunlight, a chlorophyll molecule absorbs quanta only about once every 0.1 second, while enzymes can handle their substrates in a small fraction of this time. Excitation energy migration in the photosynthetic unit provides an elegant solution to the problem of how to utilize the light-absorbing capacity of several hundred pigment molecules to feed a single enzymatic conveyor belt.

It has been asked (Franck and Teller (16)) whether enough time is available between light absorption and energy dissipation to permit effective energy transfer in the unit. If the transfer is by random walk, considerably more than 250 transfers will be needed to assure effective delivery of the quantum to the reaction center in a 250-molecule unit. (On the other hand, it may be sufficient to conduct the quantum into the neighborhood of the center, rather than to a single specific pigment molecule). The natural life-time of chlorophyll in the Chl* excited state is 15 $m\mu$ sec. (Brody and Rabinowitch (10)). With 3 % fluorescence yield (Latimer, Bannister and Rabinowitch (11)), the actual life-time is 0.5 $m\mu$ sec; with 10 % yield, 1.5 $m\mu$ sec. A thousand energy transfers during this period means a visiting time of 0.5 to 1.5 $\times 10^{-2}$ $m\mu$ sec. The width of the absorption band of chlorophyll in vivo suggests undisturbed coupling with intramolecular vibrations, and this requires electronic excitation to last $\gg 10^{-4}$ $m\mu$ sec. Comparison of the 2 figures (0.5 to 1.5 $\times 10^{-2}$ $m\mu$ sec available and $\gg 10^{-4}$ $m\mu$ sec needed) shows just about enough time for the postulated number of visits.

The range of effective migration could be extended if one could assume that fluorescence is limited by transfer of ^{1}Chl* into the metastable ^{3}Chl-state (which is plausible), and that energy migration can continue by the Wigner mechanism $^1Chl_I + {}^3Chl_{II} \rightarrow {}^3Chl_I + {}^1Chl_{II}$. Resonance transfer in the triplet state was demonstrated by Terenin and co-workers (17) in frozen hydrocarbon solutions. However, it requires an overlapping of the electron clouds of the exchange partners to make their total spin a significant invariant, and what we know about the chlorophyll monolayers in vivo may not justify this assumption.

The mechanism of energy migration considered so far corresponds to figure 1 (a), (b)—the migration of an intramolecular exciton. It may be asked whether the formation and transfer of intermolecular excitons (fig 1 (c)), with separation of charges, also is possible. This was postulated by Arnold (18) to account for light-induced electron trapping in dried chloroplast layers, and by Commoner et al (19), and Calvin et al (20), to account for light production of unpaired spins, revealed by paramagnetic resonance. However, the postulate that light absorption in the chloroplast leads to electron transfer into a conductance band, or at least, to the formation of a loosely bound intermolecular exciton, meets with difficulty. The absorption band of chlorophyll in vivo is very similar to that in vitro; this suggests that the excitation leads to the same intramolecular excited state. Simultaneous hole-electron transfer can then occur as an "afterthought" because of resonance between chlorophyll molecules. A similar delayed transfer of the electron without the hole—i.e., conversion of an intramolecular into an intermolecular exciton, indicated in figure 1 (c)—could occur only if the energy level of the second is below that of the first, which seems unlikely. (See note added in proof). It is, however, possible that charge separation occurs when the migrating exciton encounters an impurity, or a chlorophyll mole-

FIG. 5. Granular surface of a lamella (after Steinmann).

cule in a special state, which can serve as a "trap" for either the electron or the hole.

Since the quantum yields of the observed creation of trapped electrons (Arnold) and of free electron spins (Commoner, Calvin) appear to be low, these may follow exceptional absorption acts, e.g., in chlorophyll molecules immediately adjacent to traps. Furthermore, effects observed with dried chloroplasts may well be due to aggregates formed in drying. S. Brody (21) observed that illumination of crystalline chlorophyll does lead to abundant production of free spins.

Franck suggested that free electrons may be viable in the water-free "protected" parts of the chlorophyll layers, but Franck's mechanism of photosynthesis (13) does not require their occurrence.

Calvin and co-workers (20) believe that photochemical separation of charges, followed by electron and hole migration across the lamella can separate the oxidation products from the reduction products in photosynthesis. It was often pointed out by this author that this separation is the real crux of photosynthesis; in non-living photochemical processes, high energy intermediates often are produced, but tend to disappear rapidly by recombination. However, the pigment layers in chloroplasts probably are monomolecular; therefore, all that is needed to separate the oxidation product from the reduction product, is for them to be produced on different sides of this layer. They could then be drained into alternate interstices, without the need for more electron conductance than is made possible by the aromatic structure of chlorin.

Electron and hole migration in the plane of the lamella is a different matter. One could conceive of it as helping to initiate reactions in 2 different enzymatic sites—the reduction of an appropriate intermediate (such as TPN), and the generation of free O_2. However, this mechanism would be less efficient in preventing recombination; and it, too, is made unlikely by the above-mentioned spectroscopic evidence.

To sum up, we suggest, as a working hypothesis, that migration of an intramolecular exciton is the most important type of energy migration in the chloroplast, bringing excitation energy close to the photoenzymatic center, located in each, or in each few, macromolecules in the lamellae.

We still need to specify the location of the different types of chlorophyll, as well as of the accessory pigments, particularly in view of Emerson's observations (22) suggesting their distinct photochemical functions. In the case of the phycobilins, it is likely that their macromolecules also lie in the lamellae. The carotenoids (other than the photosynthetically highly efficient fucoxanthol of brown algae), must be distributed in a different way; if Platt's picture (23) of them as "electron pipelines" between excited chlorophyll molecules and electron acceptors (or donors) is correct, some carotenoids must be associated with the enzymatic reaction centers.

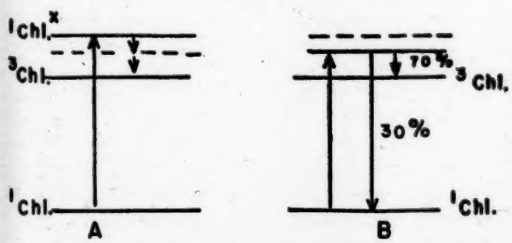

FIG. 6. Term scheme of chlorophyll in the fluorescent state, B; and in the non-fluorescent state, A. Dashed horizontal lines represent $n\pi$ levels, solid lines, $\pi\pi$ levels.

V. PHOTOCHEMICAL FUNCTION OF CHLOROPHYLL

There are other, chemical data which require fitting into the picture. These are findings by Krasnovsky and co-workers (24), Rabinowitch and Weiss (25) and Bannister (26) of the capacity of chlorophyll for reversible photochemical reduction and oxidation, suggesting that chlorophyll sensitizes photosynthesis by undergoing one, or both, of these reactions. From the studies of chlorophyll-sensitized reductions of various oxidants by ascorbic acid in pyridine, it appears (Evstigneev and Gavrilova (27), Bannister (26)), that chlorophyll is first reduced to an unstable product (a free radical?), which can reduce such unwilling oxidants as riboflavin; if not immediately reoxidized, it is then transformed into the more stable, probably valence-saturated, reduced form, pink in the case of chlorophyll a (eosinophyll). Examination of difference spectra of illuminated Chlorella cells by Coleman and Rabinowitch (28) suggested—but did not prove—that this last product may accumulate in cells—up to 1/300 of total chlorophyll when photosynthesis becomes light-saturated. The similarity of this figure and the size of the unit suggests that chlorophyll molecules associated with the reaction centers may be the ones to become reduced (perhaps, only to unstable radicals as long as no light saturation occurs, and then to saturated eosinophyll molecules).

This picture of the photophysical and primary photochemical stages in photosynthesis is speculative, and may prove wrong in parts or in toto. It seemed, however, tempting to try to develop such a picture from the data accumulated by recent research in various laboratories.

The experimental work in part utilized in this paper was carried out in the Photosynthesis Laboratory of the Botany Department at the University of Illinois by E. E. Jacobs, S. S. Brody, M. Brody, P. Latimer, and T. T. Bannister, with the assistance of the Office of Naval Research and continuous ready and invaluable help of the late Dr. Robert Emerson. The electron micrographs were obtained by A. Vatter at the Electron Microscope Laboratory of the University.

Note added in proof: Rosenberg (Faraday Society Discussion on Energy Transfer, April, 1959) pointed out that, in some molecular melts, strong photoconductivity is observed without the appearance

of a new absorption band leading to a conductance level; such conductance could result, for example, from coalescence of the metastable triplet levels of adjoining molecules, so that transfer into the triplet state, commonly postulated to precede the primary photochemical act, becomes identical with transfer into a conductance band. It remains to be seen, however, whether such processes can occur with high efficiency in non-crystalline chlorophyll monolayers.

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FURTHER STUDIES ON THE CARBON DIOXIDE BURST IN ALGAE[1,2]

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Emerson and Lewis (2) in a detailed study of the gas exchange of *Chlorella pyrenoidosa* established that immediately upon illumination there was a considerable evolution or "burst" of CO_2. When the cells were afterwards placed in the dark there was a corresponding uptake or "gulp" of CO_2 of approximately equivalent size. In their work and subsequent work of Emerson and his collaborators (6) the two-vessel manometric method of Warburg was used. A number of technical refinements were introduced into the experimental procedure so that although the method is such that small errors in pressure change can result in large errors in the calculated gas exchange, nevertheless there is every reason to believe that the method as used by Emerson revealed a new phenomenon of biological importance. Brown and Whittingham (1) subsequently made observations using the mass spectrometer which established that the gas evolved immediately after illumination was indeed CO_2. Other less specific measurements have been made using the diaferometer both with Chlorella (van der Veen (7)) and with mosses (Vejlby (9)). There is considerable evidence that the phenomenon is not confined to Chlorella although it is apparently absent from a large number of higher plants (van der Veen (8)).

The biochemical changes which result in the evolution of CO_2 in the burst are now being investigated in this laboratory using tracer CO_2. Before this technique could be profitably applied it was necessary to characterise the process so that it was certain that the biochemical measurements were made under conditions when the burst was maximal. The present paper reports observations on the effect of various factors on the physiological processes and the results in large part confirm those of previous workers.

MATERIALS AND METHODS

Chlorella pyrenoidosa Chick (Emerson strain) was maintained in pure culture and grown autotrophically in liquid medium of the composition described by Emerson and Lewis (2). A stream of air enriched with 4 % CO_2 was bubbled continuously through the cultures and cells were grown with continuous illumination for 3 to 5 days in tungsten light (30 cms distant from eight 100-watt bulbs) at 25° C. Immediately prior to measurement the culture was transferred for 20 hours to a separate bath maintained at 12° C with a much lower intensity of illumination (diffuse daylight). These conditions were found to maximize the burst in this strain of *Chlorella pyrenoidosa*. Other organisms were tested after similar pre-treatment.

Measurements were made using the two-vessel manometric method with simple Warburg manometers. Two rectangular shaped vessels were used with total volumes of 11.50 ml and 17.61 ml and with 4 ml of algal suspension. The cells were harvested, washed and resuspended in 0.05 M KH_2PO_4 solution (pH 4.6) equilibrated with 4 % carbon dioxide in air. Illumination was provided from fluorescent lights (four 40-watt Cryselco warm-white bulbs; distances varied between 15 cm and 25 cm). The steady state rate of photosynthesis was of the order of 1 to 5 times the rate of respiration. The density of the algal suspension in the manometric vessel varied between 5 and 12 %. The shaking speed of the manometer was between 200 and 250 oscillations per minute of amplitude 10 mm. Under suitably defined conditions it was found that the magnitude of the burst was proportional to the maximum initial pressure change of either vessel and for comparative studies the maximal change in the small vessel was taken as a measure of the CO_2 burst. For experiments in which comparisons of the burst in different conditions were to be made the burst was always measured following a 45 minute dark period after an initial 1st light period of 15 minutes.

RESULTS

Emerson and Lewis made observations of cells suspended either in acid phosphate solution or in culture solution. Gaffron (4) has shown that a CO_2 burst can also be demonstrated with Chlorella when the algae are suspended in bicarbonate solution. In the present work the cells were suspended in acid phosphate. Since the respiration rate is initially greater in acid phosphate than in water and shows a changing rate with time (as reported by Eny (3)) it was convenient when it was desired to maintain a more or less steady respiration rate to use very dilute phosphate or culture solution.

A number of strains of *Chlorella pyrenoidosa* were treated in the manner described and only 2 of these gave a measurable burst of CO_2 under our conditions. There were "Emerson strain 3" obtained from Emer-

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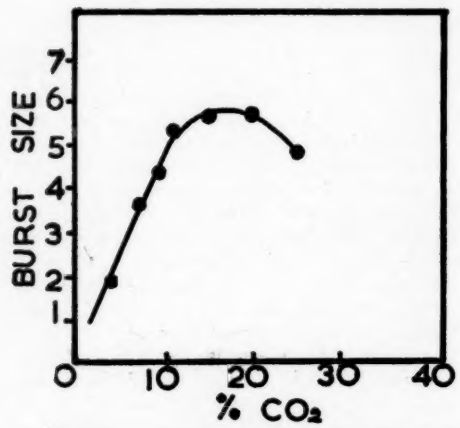
[2] This research is part of a program of work on photosynthesis generously supported by the Agricultural Research Council.
[3] The experimental work was done by one of us (R.G.H.) during the tenure of an Agricultural Research Council Studentship and as part of the work for a Ph.D. thesis at London University.

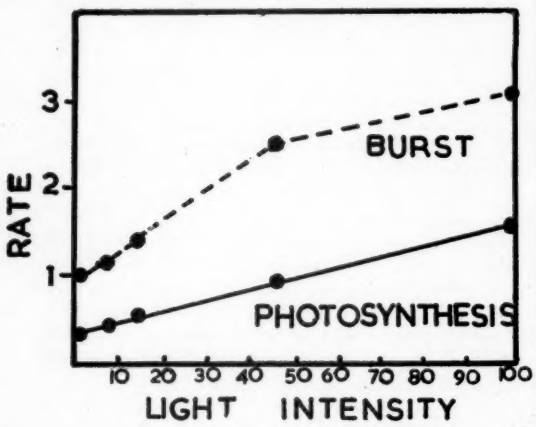
son's laboratory and "ellipsoidea" (obtained from Tamiya). However, it is our experience that if a strain has the ability to burst, then even if special pre-treatment conditions are not given, an irregularity in the gas exchange is detectable under almost all conditions. But it should be emphasized that to obtain the maximal effect a specific set of pre-treatment conditions must be given. A number of other strains of pyrenoidosa were found to give no measurable burst under the conditions investigated. The possibility remains that a survey of pre-treatment conditions might show that the pre-treatment required for maximal burst might vary from strain to strain.

The experiments subsequently described were made either with "Emerson strain 3" or with "ellipsoidea."

The influence of CO_2 pressure on the size of the burst was investigated. Increase in partial pressure above 20 % produced little further increase in size of the burst but there was a marked dependence of size of the burst on partial pressure at lower concentrations. (fig 1). In other experiments there appeared to be a depressant effect of high concentrations of CO_2 which was most marked at 50 % but could be detected in some experiments at concentrations between 10 and 20 %. As far as can be measured using a manometric technique the half time of evolution of CO_2 during the burst appeared to be independent of the partial pressure, whereas as might be expected the initial rate of uptake during the gulp is greater at higher partial pressures (fig 2).

The influence of light intensity on the burst and on the steady state rate of photosynthesis was compared. Figure 3 shows the relationship between relative light intensity and both the photosynthetic activity and the maximal burst size. Throughout the range of intensities investigated the rate of photosynthesis was directly proportional to light intensity but the burst size showed a smaller dependence upon light intensity the higher the light intensity. Some difficulties arise in investigating the higher intensities since under those conditions it is more difficult to separate the burst from the onset of a steady state of photosyn-





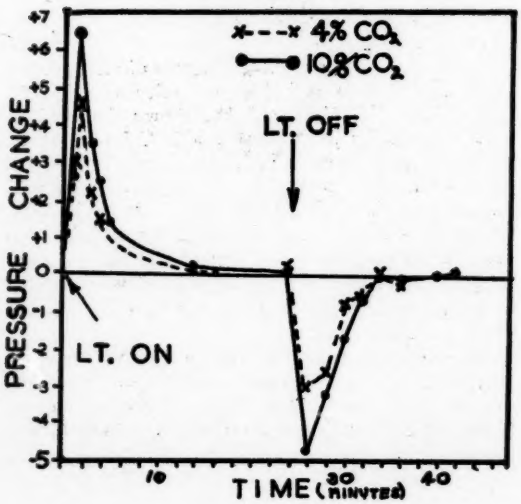


FIG. 1 (*top left*). Effect of partial pressure of CO_2 on relative burst size. 400 μl *Chlorella pyrenoidosa* (Emerson strain), pretreated for 20 hrs at 12° C in diffuse daylight measured in 4 ml 0.005 M KH_2PO_4. Ord. rate pressure change mm/min.

FIG. 2 (*bottom*). Time course of gas exchange upon illumination and darkening 360 μl *Chlorella pyrenoidosa* (Emerson strain) pretreated for 16 hrs at 12° C in diffuse daylight measured in 4 ml. 0.005 M KH_2PO_4 equilibrated with different partial pressures of CO_2. The gas exchange due to photosynthesis is minimized by the addition of 10^{-4} M iodoacetamide. All readings corrected by the respiration rate averaged for the beginning and end of the experiment. • = 10 % CO_2, X = 4 % CO_2.

FIG. 3 (*top right*). The relationship between light intensity and either the steady state rate of photosynthesis or the burst size. 140 μl *Chlorella pyrenoidosa* pre-treated as for fig 2 and measured in 4 ml 0.005 M KH_2PO_4 equilibrated with 9 % CO_2. Neutral glass filters (Chance glass ON32, ON31) used to adjust light intensity.

thetic activity. There is therefore a measure of uncertainty in the calculated size of the burst but it is clear that the burst approaches saturation at a lower intensity than that required to saturate photosynthesis.

When the temperature for measurement was varied between 8.5 and 19° C there was little effect on the burst, either on the half time of the burst or with less certainty, on the size of the burst. Van der Veen (7) made observations at higher temperatures and suggested that the CO_2 burst is smaller at temperatures as high as 21 to 26° C. On the other hand in our experiments on the uptake of CO_2 in the gulp, the half time was appreciably longer the lower the temperature. This would be consistent with the view that the burst is determined primarily by a photochemical reaction but that the gulp is thermochemical.

Attempts to distinguish between the burst and photosynthesis on the basis of inhibitors, previously made by Emerson and Lewis and Brown and Whittingham, have been extended in the present work. The finding of Brown and Whittingham that *o*-phenanthroline inhibited photosynthesis to a far greater extent than the CO_2 burst was confirmed using a concentration of phenanthroline of 10^{-4} M. Similarly 10^{-4} M iodoacetamide almost completely inhibited photosynthesis while having no measurable affect on the burst. A 2nd type of inhibitor decreased the size of the burst while having little effect on the steady state of photosynthesis. Brown and Whittingham found cyanide to be of this type and the present work showed that "Dieca" at a concentration of 10^{-3} M behaved in a similar way. Other inhibitors such as maleic acid (10^{-2} M) inhibited the burst and photosynthesis to about the same extent. Fluoride (M/10) was found to have little effect on the burst or photosynthesis.

Discussion

The production or burst of CO_2 immediately after illumination has been observed only with a limited number of organisms and with certain individual strains of a selected organism. It is difficult to detect a burst if the induction phase of photosynthesis is short. Thus the burst is not easily observed either with a high light intensity or at higher temperatures. The measurement of a burst is most conveniently made when conditions are such that the induction phase is a certain minimum length. Thus until a wider range of conditions have been investigated than at present, it is not possible to exclude the existence of a CO_2 burst as a general feature of all photosynthetic organisms.

Observations by van der Veen on higher plants using a diaferometer showed that there is frequently a marked initial uptake of CO_2 followed by a relative cessation of uptake and then a final continuous acceleration to the steady state rate. The relationship between the initial uptake phenomenon and the CO_2 burst in Chlorella has not yet been fully elucidated. Massinni has investigated the effect of inhibitors on initial uptake and suggested on the basis of those results that the initial uptake is closely related to the development of phosphorylative activity. He also found that the initial uptake was inhibited by phenanthroline and points out that this differentiates it from the CO_2 burst which Brown and Whittingham showed was relatively insensitive to phenanthroline. On the other hand iodoacetamide inhibits photosynthesis but has little effect on the initial uptake; the same was found to be true for the burst in the present work. Massinni (5) also found that a higher partial pressure of CO_2 and a lower light intensity were required to saturate initial uptake as compared with photosynthesis. The same is true for the CO_2 burst as shown in this paper and indeed there is good agreement between the results of Massinni concerning the effect of these factors on initial uptake and our results relating these factors to the burst. With a measuring technique of rapid time response it might be possible to show in Chlorella that photosynthesis actually commences prior to the burst. If this were the case the burst would then appear as a depression in the time course for CO_2 uptake and resolve the curve into 2 sections of an apparent initial uptake and the development of a steady state rate. On this view the factors responsible for the apparent initial uptake would be the same as those responsible for the burst and in the absence of a burst there would be no depression in the CO_2 uptake curve. But the real initial uptake would be determined by the same factors as control steady state photosynthesis. Massinni's observations showing that apparent initial uptake was inhibited by phenanthroline depended on observations at a single concentration and until measurements have been made over a range of concentrations it would be premature to believe that this is the basis of a fundamental distinction. It may be that the effect of a single concentration of phenanthroline giving the same percentage of inhibition for photosynthesis and for initial uptake observed by Massinni was simply fortuitous. So far no observations have been made on the time course of CO_2 uptake following the cessation of illumination in higher plants comparable with the gulp in Chlorella.

Warburg has also demonstrated a CO_2 uptake in Chlorella which involves the carboxylation of γ-aminobutyric acid to form glutamic acid. This reaction became most easily observable in the presence of fluoride or after a period of anaerobic conditions. As Emerson and Lewis showed, the burst phenomena are absent in Chlorella after exposure to anaerobic conditions and this has been confirmed by subsequent workers. The present paper also shows that fluoride has no effect on the phenomena measured in this investigation.

The plant contains a number of carboxylation systems and it is probable that many of these will adjust to a new steady state after the onset of illumination. The adjustment resulting from the onset of illumination might be attributed to either a general or a specific consequence of light action. It has been frequently suggested that the onset of illumination results in a change of pH within the cell and that this may in itself result in the evolution or uptake of CO_2. If

such an explanation is accepted it follows that the mechanism responsible for the change in pH cannot be one which obligatorily results in photosynthesis. This view is based not only on the differential inhibition of the burst and photosynthesis but also on the fact that the influence of external factors on the 2 processes is distinct. Thus the burst shows a greater dependence on CO_2 partial pressure than does photosynthesis and it is saturated by a lower light intensity than is photosynthesis. It follows that a biochemical explanation of the burst is probably not to be sought in transients of compounds directly concerned in the photosynthetic cycle. These compounds will undergo changes in the light but if the compounds which are more directly related to the burst are to be easily distinguishable it is of the greatest importance that investigations concerned with the burst should be made under conditions where the burst is maximized and the steady state photosynthetic activity minimized.

Summary

1. Two out of five strains of Chlorella have been shown to exhibit a CO_2 burst and gulp of the type reported by Emerson and Lewis after suitable cultural conditions.

2. The burst has been measured using a manometric technique and its dependence on partial pressure of CO_2 and relative light intensity determined. The burst approaches saturation compared with steady state photosynthesis at higher partial pressures of CO_2 and lower light intensities.

3. Iodoacetamide and *o*-phenanthroline at concentrations of 10^{-4} M both inhibited photosynthesis to a greater extent than the burst. Other inhibitors affected the burst to a greater extent than photosynthesis.

4. The effect of these factors on the burst is discussed and compared with observations by other workers on the initial uptake of CO_2 observed in higher plants.

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COMPLEXITIES OF MANOMETRIC MEASUREMENT OF CHANGING RATES OF GAS PRODUCTION [1]

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The occasion calls for some introduction to this paper. Some years ago we at the Botany School, Cambridge, had the privilege of having Robert Emerson as our guest for 6 months. As he has indicated (1) we discussed diffusion lag in manometry, a problem I had tackled for one of my research students in 1935 (2). We planned to write together at some time a full paper with experimental illustrations. In 1955 he wrote saying that he had had to postpone his promised visit for the above purpose and added in his customary style "I can think of plenty of reasons for this, but maybe they are all smoke screens behind which I hide my real worry that your equations make my last 3 years of experimental work look rather silly." Without him, and in the short time available, I have been able to put but little flesh on the skeleton of equations. Given his help it might have lived.

The net rate of passage of gas from the liquid phase to the gaseous phase in a Warburg manometer vessel is equal to $e(x_l - \sigma x_g)$, where x_g is the concentration in the gaseous phase, x_l that in the liquid, and σ is the solubility of the gas in the liquid so that at equilibrium $\sigma = x_l/x_g$. The value of e will increase with effective area of the interface, decrease with increase of effective thickness of layer of shearing of liquid and of gaseous phase, decrease with increase of the energy of activation necessary for a gas

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molecule to pass from liquid to gaseous phase. The speed of shaking and the shape of the vessel will affect all except the last. The actual area will vary with time and the actual thickness of layers of shearing will vary with time and place. Unless the layer of shearing in the gaseous phase is great compared with that in the liquid, which is very unlikely, then since the coefficient of diffusion in the gaseous phase is 10^4 times that in the liquid this layer of shearing can be neglected. Then, if the energy of activation is relatively small, e is the product of the effective area of interface and the coefficient of diffusion in the liquid phase divided by the effective thickness of the layer of shearing in the liquid.

If ρ is the rate of production of a gas in the liquid phase then

$$\rho = v_g \frac{dx_g}{dt} + v_f \frac{dx_f}{dt}$$

where v_f and v_g are the volumes of the phases.

The rate of appearance in the gaseous phase, g, is given by

$$g = e(x_f - \sigma x_g) = v_g \frac{dx_g}{dt} \quad [2]$$

Hence $$\frac{dg}{dt} = \frac{e}{v_f}\left\{\rho - \frac{g(v_g + \sigma v_f)}{v_g}\right\}$$

If ρ is constant then eventually $\frac{dg}{dt}$ will become zero and ρ can be obtained by multiplying g by a constant $k = \frac{v_g + \sigma v_f}{v_g}$; the usual procedure. If however ρ is not constant then from a curve of g against time $\frac{dg}{dt}$ can be determined and ρ can be obtained by adding

$$\frac{v_f}{e} \cdot \frac{dg}{dt} \quad \text{to } k \cdot g.$$

This requires a knowledge of e.

If the relationship between ρ and t is known then by solution of the differential equation the relation between g and t can be obtained. For example, if, after a time with ρ constant long enough for g to have approached very close to ρ/k, the rate is suddenly increased to $\rho + \Delta\rho$ then the relation between Δ g, the difference between the new value of g and the old, and time is given by

$$\Delta g = \frac{\Delta\rho}{k}\left\{1 - \exp(-ekt/v_f)\right\}$$

Hence the value of e can be determined from the slope of the line of $\ln(\Delta g_\infty - \Delta g)$ against time. With fixed experimental conditions the tempo at which Δg approches its final value, $\frac{\Delta\rho}{k}$, will vary from gas to gas not only because of variation of e, to which reference has already been made, but also because of the variation of k with σ.

As the achievement of a change of ρ, such as that indicated above, may be difficult it is well to consider another method of determining e.

If no gas is being produced in the liquid phase and this is in equilibrium with the gaseous phase then a sudden change in composition of the gaseous phase will result in an exchange of gases between the two phases with a consequent change of pressure. If two gases are involved, say oxygen and nitrogen, then the tempo of equilibration will be complex because e and k are different for the two gases. If a liquid freed from all gases is suddenly exposed to an atmosphere of pure gas, then the rate of change of the concentration in the gas phase, x_g, is given by

$$-\frac{dx_g}{dt} = \frac{e}{v_g}\left\{\sigma x_g - ({}_o x_g - x_g)\, v_g/v_f\right\}$$

where ${}_o x_g$ is the original value of x_g.

On integration this gives

$$x_g - {}_\infty x_g = ({}_o x_g - {}_\infty x_g)\exp(-ekt/v_f)$$

Having determined e for oxygen it is possible to determine the course of rate of oxygen production subsequent to the moment of illumination of a suspension of algal cells in a carbonate-bicarbonate buffer during a period when the change of partial pressure of CO_2 is negligible. The essentials are a curve of pressure against time from which g, the rate of change of oxygen in the gaseous phase, can be obtained. Then with the value of e for oxygen determined as above $\frac{v_f}{e} \cdot \frac{dg}{dt}$ is added to kg to give ρ, the rate of production of oxygen.

When the two-vessel method is used to determine transients of carbon dioxide as well as oxygen we have the following equations, where capitals refer to one flask and lower case to the other and subscripts 1 to oxygen and 2 to carbon dioxide.

$$\frac{dg_1}{dt} = \frac{e_1}{v_f}(\rho_1 - k_1 g_1) \quad \text{and} \quad \frac{dg_2}{dt} = \frac{e_2}{v_f}(\rho_2 - k_2 g_2)$$

$$\frac{dG_1}{dt} = \frac{E_1}{v_F}(\rho_1 - K_1 G_1) \quad \text{and} \quad \frac{dG_2}{dt} = \frac{E_2}{v_F}(\rho_2 - K_2 G_2)$$

Since only net rates of change in the gaseous phases, $g_1 + g_2$ and $G_1 + G_2$ are known we cannot obtain ρ_1 and ρ_2 without further information.

If we know how the rate ρ_1 and ρ_2 changed with time then the 4 equations can be integrated. For example if

$$\rho_1 = R_1\left\{1 - \exp(-\nu_1 t)\right\}$$

$$\text{then } g_1 = \frac{R_1}{k_1}\left[1 - \frac{e_1 k_1}{e_1 k_1 - \nu_1 v_f}\exp(-\nu_1 t) + \right.$$

[2] When h is the rate of pressure change with a Warburg manometer,

$$\frac{\sigma(v_v + \sigma v_f)}{v_g} = \frac{h(v_v \frac{T}{273} + a v_f)}{P_o}$$

a = solubility of gas expressed as volume of gas (at N.T.P.) dissolved in unit volume of liquid when in equilibrium with a partial pressure of gas equal P_o (760 mm Hg).

$$\frac{\nu_1 v_f}{e_1 k_1 - \nu_1 v_f} \exp(-e_1 k_1 t/v_f)\Big]$$

if ν is known for both ρ_1 and ρ_2 we should have 2 equations

$$G_1 + G_2 = \frac{R_1}{K_1} a + \frac{R_2}{K_2} \beta$$

$$g_1 + g_2 = \frac{R_1}{k_1} a' + \frac{R_2}{k_2} \beta'$$

where a, β, a', and β' stand for the respective bracketed terms. These equations can be solved for R_1 and R_2. If ρ_1 and ρ_2 vary with time in an unknown way then a, β, etc. cannot be evaluated and we cannot solve the equations.

If the lag due to resistance to interchange between gaseous and liquid phases is ignored, that is a, etc. are assumed to be unity, and the value for ρ_1 calculated as c_1.

$$c_1 = \frac{K_2(G_1 + G_2) - k_2(g_1 + g_2)}{K_2/K_1 - k_2/k_1}$$

$$\text{then } c_1 = \frac{R_1(aK_2/K_1 - a'k_2/k_1) + R_2(\beta - \beta')}{K_2/K_1 - k_2/k_1}$$

If subsequent to illumination oxygen production proceeded at a steady rate ρ_1 and carbon dioxide was consumed at the same steady rate, that is $\rho_2 = -\rho_1$, then R_1 and R_2 in the above equation are replaced by ρ_1 and $-\rho_1$ and

$$a = \left\{ 1 - \exp(-E_1 K_1 t/v_F) \right\}$$

$$a' = \left\{ 1 - \exp(-e_1 k_1 t/v_f) \right\}$$

$$\beta = \left\{ 1 - \exp(-E_2 K_2 t/v_F) \right\}$$

$$\beta' = \left\{ 1 - \exp(-e_2 k_2 t/v_f \right\}$$

and c_1 would be an underestimate of ρ_1 at least during the early stages of equilibration lag. If the vessels were paired for oxygen (1) then $a = a'$ and

$$c_1 = \rho_1 \left\{ a - \frac{\beta - \beta'}{K_2/K_1 - k_2/k_1} \right\}$$

If, however, there was a big enough burst of CO_2 during this period then c_1 could exceed β_1. That is there could be an apparent burst of oxygen.

Sufficient has been said to show that estimates of rates of gas production from manometer readings over a relatively short period, subsequent to a change of conditions which result in a change in rate, can be misleading.

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RELATION BETWEEN RESPIRATION AND PHOTOSYNTHESIS IN THE GREEN ALGA, ANKISTRODESMUS BRAUNII[1]

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In what way does light affect the respiration of photosynthetic cells? In more than a century since the reaction of photosynthesis was identified in its overall aspect this question has been asked repeatedly. The earliest concern was a purely kinetic one in that "apparent" photosynthesis required correction for respiration if "true" photosynthetic rates were to be calculated and measured as a function of environmental parameters. The possible influence of light on the photosynthetic rate again became of paramount importance when quantum yield determinations were made on photosynthesis beginning some 30 years ago. Encouraged by what indirect evidence was available, investigators of photosynthetic efficiency generally have come to rely on the assumption that, if rates of dark metabolism were the same before and after a light period, then during illumination this same respiratory rate could be used for correcting the rate of apparent photosynthesis.

Less than 20 years ago the basic question was posed not in kinetic but in chemical terms. Thimann (18), in a speculative note, asked in effect if photosynthesis could not be the reverse of respiration in respect to its intrinsic mechanism as well as in its overall equation. Since then our rapidly expanding biochemical knowledge of respiratory pathways has several times called for a rephrasing of the question. The existence of chemical intermediates common to both photosynthetic CO_2 reduction and to glycolysis and respiration made it difficult to feel complacent about their kinetic independence. Also, within the last decade, has come the recognition that at least some coenzymes and electron carriers, ubiquitous in the catabolic schemes which modern biochemistry uses as working models, may be

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photochemically reduced by preparations from green cells and furthermore that the pyridine nucleotide pools of chemical potential energy, so interrelated with anaerobic and aerobic stages of respiration, are subject to similar photochemical regeneration. All this makes it seem rather unlikely that strict kinetic independence of respiratory and photosynthetic gas exchanges should in most cases obtain, and the kineticists' assumption that respiration and photosynthesis proceed almost without reciprocal influence (except in relatively long term experiments) is hardly to be anticipated from modern biochemical considerations. In any case, the opposing processes of respiration and photosynthesis cannot very well employ potentially rate controlling reaction steps in common without there being a most intimate dependence of the rate of the one on that of the other. Should this dependence not be observed, we are almost compelled to assume either that the mechanisms are truly chemically independent or that they chiefly operate in separate morphological compartments of the cell. Since we are dealing with systems which are heterogeneous in both gross and fine aspects, perhaps whatever kinetic independence of photosynthesis and respiration may be observed is but the fortuitous consequence of a cellular morphology which confines the photosynthetic apparatus to organelles which account for only a minor fraction of the cell's respiratory activity.

Whether or not biochemical compartmentalization on morphological grounds needs to be invoked can only be determined from kinetic observations, and the basic question—to what extent does light affect the respiratory rate of photosynthetic cells?—is one to be settled only by quantitative observations. Also, physiological variation between species being what it is, an answer to the question for one plant may not form a secure basis of generalization.

Recognizing that in the respiratory process as well as in photosynthesis gas exchange quotients may depart from unity, it seems that an unequivocal answer, if it can be given at all, must be provided with respect to both metabolic gases simultaneously. Also, if the answer is to be direct, it appears that the only method for simultaneous measurement of production and consumption of a gas within a single experimental system is by the employment of isotopic tracers.

The essential contribution made possible by the use of tracers is just this separation of concurrent processes of consumption and production of the same gas. Throughout the present paper, when we speak of gas *consumption*, we are referring to that process alone without regard to whether the same gas also is being produced. Similarly *production* refers to the evolution of gas whether or not gas uptake occurs as well. These terms thus do not have here the overall characteristic which is conventional in considerations of *net* gas change.

Some tracer work having a direct bearing on this matter already has been published. It was shown in this laboratory, for *Chlorella pyrenoidosa* Chick. and for some other photosynthetic species, that illumination at low to moderate intensities had only minor effects on the endogenous respiratory rate insofar as O_2 was concerned (2). These results, obtained under a necessarily limited range of experimental conditions, strongly suggested that respiratory and photosynthetic processes functioned quite independently. Nevertheless evidence from tracer studies was by no means unilateral. In certain species, cases of extreme photoinhibition or of moderate photostimulation of tracer O_2 uptake were observed (4, 13). Results from radiocarbon labelling experiments by Calvin and associates (1, 5) were interpreted as evidence that the Krebs cycle does not operate in bright light and, from kinetic measurements with tracer CO_2 (21), it was concluded that CO_2 evolution was reduced by intense light to about one half its value in darkness. This latter finding was of special interest when contrasted with measurements using tagged O_2 (19) on the same kind of plant (barley) and under rather similar conditions; there a marked photoinhibition of O_2 was not observed.

While the various pieces of direct experimental evidence are somewhat fragmentary, it is tempting nevertheless to assume that CO_2 production is mediated in photosynthetic cells by oxidative decarboxylations such as occur in the Krebs cycle or the pentose phosphate cycle, to accept at face value the purported photoinhibition of CO_2 production and to reconcile the reduced CO_2 evolution with the relatively unchanged O_2 consumption by proposing a substitution, during the light, of photochemically generated reductant for the "substrate hydrogen" which serves alone to reduce O_2 in dark metabolism. Speculating that photosynthesis and respiration are related in this manner, we may examine the salient features of a general scheme illustrating the postulated interrelation and determine what consequences of its operation ought to be observable. Such a model is shown in figure 1. Much more detailed schemes could be employed suggesting possible interplay between breakdown and synthesis through, let us say specifically, control of the DPN^+/DPNH ratio (leading to enhanced respiratory activity in the light), or through a downward adjustment of the ADP/ATP ratio by photophosphorylation (to produce respiratory inhibition), or perhaps even more directly via regulation of the concentration of a rate limiting common intermediate such as phosphoglyceric acid. However, our limited kinetic as well as biochemical information about the systems with which we are dealing hardly justifies a model more detailed than is necessary for illustrative purposes. The operation of the model (fig 1) is as follows:

If no interrelations between respiratory and photosynthetic metabolism are postulated except those arising from the existence of a common pool of compounds which collectively represent the ultimate respiratory substrate and at the same time the ultimate sink for the organic products of photosynthesis, then only the solid lines in figure 1 apply. Respiration and photosynthesis are seen as independent processes. Photochemically produced reductant is then accounted for quantitatively by CO_2 assimilation (reaction 1). On

the other hand, if photochemically generated reductant is capable of reducing directly (or, more likely, after its reducing potential has been degraded to more or less the appropriate level) any of the respiratory components as indicated by the dashed lines, then photosynthesis must, at least under some conditions, affect respiration. If the reductant, generated photochemically, is capable of reducing O_2 directly (reaction 2), this would tend to increase O_2 consumption in the light but would not alter the rate of respiratory CO_2 production. If the reductant can react directly with one of the cytochrome chain components (reaction 3), it would tend to stimulate O_2 consumption via the respiratory oxidase system. If the carrier-oxidase chain were running at capacity, substitution of photochemically produced reductant for the "substrate hydrogen" normally serving as respiratory reductant would necessitate a decrease in the rate of oxidative decarboxylation. In consequence CO_2 production would be reduced. If, by photochemical reduction (reaction 4), the pyridine nucleotide redox potential should be forced so low that coenzyme molecules cannot easily be loaded with "respiratory" electrons, similar inhibition of CO_2 evolution (accompanied by unaltered or even stimulated O_2 consumption) would be expected. If the glycolytic intermediate, phosphoglyceric acid, were to be reduced directly (reaction 5), conceivably this could limit the supply of pyruvate to the Krebs cycle so that, in strong light, slowing down of the cycle might lead to reduced O_2 uptake as well as a proportionate inhibition of CO_2 production.

Figure 1 is of course not the only model which might be suggested to illustrate possible light effects on respiratory processes. However other models lead to the same or similar expectations. They have in common the feature that, if photosynthesis and respiration are related through the photochemically produced reductant, this relation may become apparent either as a light induced deficiency in respiratory CO_2 production or as enhancement of O_2 consumption or both. Assuming for convenience that the quotients of both respiration and photosynthesis normally are unity, then the rate of photochemical generation of reductant corresponds stoichiometrically to the rate of O_2 evolution. The operation of any of the reactions 2, 3, 4, or 5 in figure 1 will account for the fate of some of the reductant by a path other than CO_2 reduction (reaction 1). The rate of photosynthetic reduction of CO_2 would diminish and the respiratory portion of the gas exchange would be altered—effects which would be detectable as enhanced O_2 consumption or reduced CO_2 production or both. These effects should bear a stoichiometric relation to the inhibition of photosynthetic CO_2 uptake rate.

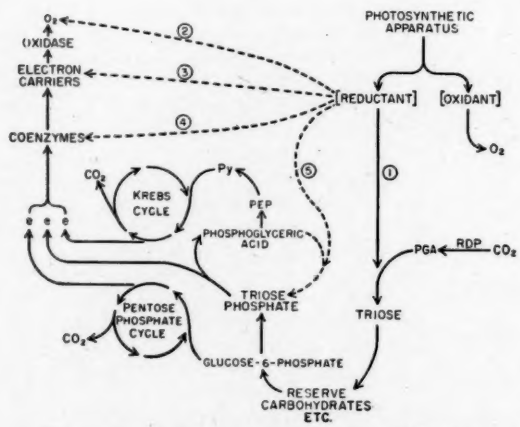

FIG. 1. Schematic representation of possible influences on respiratory processes by means of a reductant produced by a partial reaction of photosynthesis.

If, instead of a reductant, it is the photochemically produced oxidant (vide Gaffron, (10)) which is postulated as influencing the respiratory rate, any component with which it reacts—directly or indirectly—must become oxidized, and in such a model, analogous with figure 1, it is readily shown that the influence of light would be to depress both production and consumption of O_2 and to accelerate CO_2 evolution—changes in respiratory gas exchange which are opposite from those predicted above. Only when O_2 uptake *and* CO_2 *evolution* remain unchanged in the light can we conclude that photosynthesis and respiration are truly kinetically independent.

What has been said in qualitative terms about the interrelations suggested by figure 1 may be made quantitative as follows: Let P_{CO_2} and U_{CO_2} designate respectively rates of production and uptake of CO_2. Similarly P_{O_2} and U_{O_2} are rates of production and consumption of O_2. In the light an increase in O_2 uptake rate as compared with the dark value is referred to as ΔU_{O_2} and a decrease in CO_2 evolution as ΔP_{CO_2}. For every 4 units (electron equivalents) of photo-reductant formed in the light there must be generated 4 units of oxidant corresponding to the production of 1 O_2 unit. The 4 units of reductant may be accounted for by the uptake of 1 unit of CO_2, or by the enhancement of U_{O_2} to the extent of 1 unit, or by the depression of P_{CO_2} to the extent of 1 unit, or by any combination of U_{CO_2}, ΔP_{CO_2}, and ΔU_{O_2} which adds up to 1 unit. Thus in the light according to our model,

$$P_{O_2} = U_{CO_2} + \Delta P_{CO_2} + \Delta U_{O_2} \qquad (1)$$

Another way of looking at the matter is to say that only as long as equation 1 is obeyed will the chemical reduction level of the cell remain unaltered.

The purpose of the present study was to test the validity of equation 1 by evaluating its 4 component terms simultaneously for each of a series of experiments. These experiments were carried out over a range of light intensities both above and below photosynthetic compensation. Agreement of the data with equation 1 implies consistency with the general model shown in figure 1.

Materials and Methods

In a series of contributions from this laboratory, respiration rates of photosynthetic cells have been measured in the light by a method which employed tracer O_2 in the gas phase of the experimental vessel. By thus tagging the environmental O_2 consumed by the cells, the rate of O_2 uptake was measured independently of the photosynthetic production of unlabelled O_2 from water. In principle, analogous experiments can be performed with tracer CO_2. However, in preliminary studies carried out several years ago, CO_2 isotope exchange in the dark was observed to be of such a magnitude that large corrections for this exchange were required (18). The uncertainty of these corrections rendered the interpretation of results obtained with tracer CO_2 much more difficult than in the case of tracer O_2 experiments where isotope exchange in the dark was not detectable under the conditions prevailing in biological experiments. Numerous studies carried out subsequent to our earlier publications and on divers photosynthetic species have demonstrated that CO_2 isotope exchange is not as serious a drawback to the use of the tracer method as we at first thought, for it has been found that the earlier results showing excessive CO_2 exchange were atypical; high exchange rates have since been the exception rather than the rule. In short, we usually are able to effect a kinetic separation of simultaneous CO_2 uptake and production without prohibitive corrections for isotope exchange. Fortuitously, certain second order corrections which apply to such isotope data (to be described below) are even of much smaller magnitude with tracer CO_2 than with tracer O_2 measurements.

The organism chosen for the present work was *Ankistrodesmus braunii* (Naegeli) Collins. This is a green alga similar morphologically and physiologically to the genus, Scenedesmus. The cells were cultured autotrophically in medium V of Norris et al (15) at 26 to 29° C. Culture flasks, illuminated from below by a bank of white fluorescent lights with a red-orange filter (Corning, no. 348) were agitated by a stream of air enriched with 5 % carbon dioxide and by occasional shaking. Cultures were harvested after 3 to 5 days while the growth rate was still high. Cells collected by slow centrifugation (< 1000 G) were washed once and were resuspended in media to be used in the experiment. Ordinarily this was 0.05 M potassium phosphate buffer (pH 5.8) containing 2 % glucose.

A Nier-Consolidated mass spectrometer (Model 21-201) continuously monitored the gas phase of the reaction vessel which was a rectangular Warburg-type vessel of 15.8 ml total volume when attached to the joint which was in turn connected to the mass spectrometer inlet leak. The design of this joint and the method of shaking the vessel in a constant temperature water bath did not differ in any essential manner from what was illustrated previously (13). The use of the mass spectrometer as adapted for the required measurements also has been described (3). Labelled O_2 and labelled CO_2 were introduced initially into the gas phase of the experimental vessel and continuous monitoring of the gas phase with the mass spectrometer was carried on thereafter.

When cells were suspended in inorganic buffer only, the respiratory rate was at first high but declined over several hours to a small fraction of the initial rate. Addition of 2 % glucose to the experimental medium prevented this decline. Since the smaller the respiratory rate the greater was the percent error in its measurement, data on glucose stabilized respiration was more reliable than were measurements on endogenous gas exchange. Also it was convenient to work with cells whose dark metabolism was not changing greatly with time. Therefore, critical light-dark comparisons were made only with cell suspensions containing glucose.

For present purposes it is necessary to reconsider the principles involved in the tracer method of measuring independently both uptake and output of a given gas. Whether O_2 or CO_2 is considered, the presence of isotopically enriched gas in the environment insures that gas produced will have an isotopic composition different from that of gas consumed. If isotopic enrichment is large, it is possible to ignore the influence of the small natural concentrations of rare isotopes of O_2 and of CO_2. It is readily seen that the actual rates of production, P, and of consumption, U, are functions of the rates of metabolism of both isotopes. These "true" rates are given by the equations,

$$P = h\,(R - R^* \frac{M}{M^*}) \qquad (2)$$

$$U = h\,R^*\,(1 + \frac{M}{M^*}) \qquad (3)$$

where R and R* are rates of change of the 2 isotopic forms of the gas being metabolized, M and M* are the mean concentrations of the respective isotopes which prevail during the period of measurement at the site of gas consumption, and h is a dimensional constant. While it is approximately correct to consider U a linear function of R*, as was done in some of the earlier work from this laboratory, when only relative rates of gas exchange are required, it should be kept in mind that this simplification is valid only as long as M/M* remains essentially constant during the experiment. This ratio, measured in the gas phase, can be expected to remain adequately constant only if the total tension of the gas is relatively high and the rate of change of either isotopic form of the gas relatively low.

Furthermore, it is the ratio, M/M*, at the site of reaction which is critical rather than the ratio which prevails in the gas phase where the mass spectrometer can measure it. Under any single set of steady state conditions a difference in this ratio on passing from the gas phase to the reaction site is of minor consequence for relative measurements. But, if it is necessary to compare data taken under different steady state conditions (viz. light vs. darkness), the M/M* ratio at the reaction site can be appreciably different under the separate conditions. With photosynthesiz-

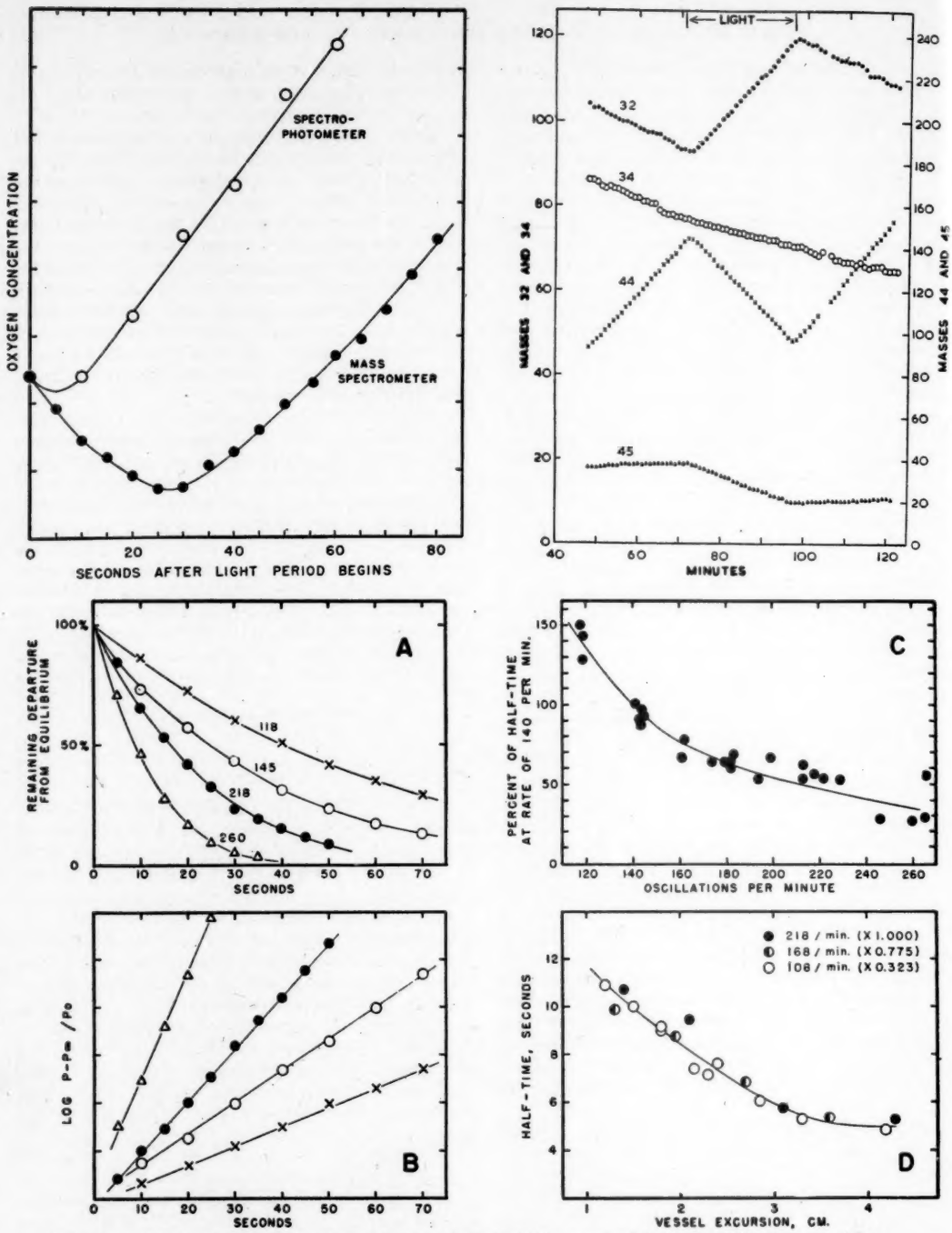

FIG. 2 (*top, left*). Apparent induction period for photosynthesis measured in liquid and in the gas phase.

FIG. 3 (*below*). A. Time course of equilibration of O_2 in a rectangular Warburg type vessel (15.8 ml) containing 3 ml 0.1 M KH_2PO_4. Gas phase: He, O_2, H_2O vapor. The parameter of the 4 curves was shaking rate as indicated in oscillations per minute. B. Semilogarithmic plot of data above. C. Relation between equilibration and shaking rates. D. Relation between equilibration rate and vessel excursion distance for 3 different shaking rates. Ordinate values were multiplied by the factors shown so as to superimpose curves.

FIG. 4 (*top, right*). Example of mass spectrometer data for 2 isotopic forms of CO_2 (masses 44 and 45) and 2 of O_2 (masses 32 and 34). Expt. 2607. 8.4 μl cells. 26.0° C. Gas phase, CO_2 : O_2 : He (1 : 4 : 328). Tungsten illumination through red filter (Corning no. 2403) at intensity corresponding to 2.6 times compensation.

ing cells the ratio will be smaller in the gas phase than at the site of reaction so that the O_2 utilization rate as calculated by equation 3 must be considered a minimal value; the true rate may be higher. A qualitative way of looking at this discrepancy between calculated and true rates is to think of O_2, as it is evolved but before it can escape into the gas phase, being utilized by the respiratory enzymes in preference to the O_2 of the gaseous environment which must cross a significant diffusion barrier before the enzymes have access to it. The use of the tracer method for experiments such as ours has been criticized along these lines by Warburg (20). Decker (6) has also raised objections which are more easily considered in these terms.

The difference between M/M* ratios in the gas phase and at the site of reaction can be minimized by various devices but never eliminated and, when the difference is large, it leads to what has been termed "preferential reutilization" of the gas produced (2). In previous studies, keeping the metabolic rate low and the prevailing tension of the gas being measured relatively high (insofar as technical considerations would permit) offered assurance only that the errors in relative metabolic rate determinations were as small as possible. It now seems that errors from this cause were sometimes larger than we had thought and, when either more precise or absolute measurements are needed, corrections for "preferential reutilization" usually are mandatory.

In the present work, absolute rather than merely relative measurements were required. Also, as can be seen from inspection of equations 2 and 3, computed changes in production or consumption of the 2 metabolic gases depended upon 4 essentially simultaneous measurements of concentrations of different mass components of the gas phase and upon 4 measurements of the rates of change of these components as compared with the result of similar computations from a comparable set of 8 experimentally determined values taken from another condition being tested. Thus the conclusion to be arrived at from a single experiment (e.g., an individual point in fig 7) is sensitive to error in any of 16 items of data. Under such circumstances the necessity to reduce all known errors to a minimum was unusually compelling. Therefore corrections of M/M* in equations 2 and 3 have been introduced so that the computed values approach as closely as possible the ratios prevailing at the site of reaction. If these corrections are ignored, when relatively high metabolic rates are determined by the tracer method, the estimated rates can be in error by over 100 % in the case of O_2 at least. This error arises from the interrelated effects of diffusion gradients which exist in the experimental system and the corrections are based on an approximate evaluation of these gradients.

Mixing within the liquid is so rapid in a well shaken vessel that appreciable diffusion gradients are not established within the liquid proper and, for several reasons, it seems likely that, of the various diffusion barriers which must exist between gas phase and enzyme, that which is by far the most important exists at the liquid-gas interface (shearing layer) in the reaction vessel.

The effect of this barrier alone is illustrated in figure 2 in which time course data from gas phase mass spectrometer measurements are compared with liquid phase determinations obtained by a different method (21). Chlorella cells were utilizing O_2 in the dark until, at zero time, the suspensions were illuminated. The much greater response lag in the case of the mass spectrometric data is attributable almost entirely to the diffusion gradient across this barrier for which we must make correction.

Provisionally, we shall assume that with small cells the barrier at the cell-liquid interface generally is unimportant in comparison with the barrier which exists at the gas-liquid interface.

The effects of the interfacial barrier in manometric experiments have been considered both theoretically and experimentally by Roughton (16) and by Myers and Matsen (14) in terms of the usual stationary film theory. Only a brief explanation is in order here. The influence of the barrier can be described quantitatively by an equilibration constant, K, which characterizes the rate at which a concentration difference spontaneously disappears across the interfacial film according to the equation,

$$(p - p_\infty) = e^{-Kt} \qquad (4)$$

in which p is the partial pressure of the gas at any given time, t, and p_∞ is the final value of p after equilibration. The constant, K, is a function of the chemical nature of the 2 phases, the surface area, the amplitude and frequency of shaking the vessel, and the volumes of liquid and gas space.

In order to determine K experimentally, one makes a sequence of measurements of the partial pressure of a gas which is undergoing equilibration across the interfacial diffusion barrier. Plotted semilogarithmically, the data on departure from equilibrium are linear with time. The value of K which applies to the conditions under which the measurements were made can be computed readily from the slope of this linear plot as described by Roughton. If desired, values of K are easily converted to the half-times for gas phase response to liquid phase changes—the familiar way of expressing equilibration lag. Using this procedure variables such as vessel shape, liquid volume and composition, presence of surface active materials, gas phase composition, vessel shaking rate, and excursion distance were studied systematically to determine their repective influences on K—i.e., on equilibration lag. Figure 3 illustrates by example this method of observing effects of the last two variables mentioned.

When making measurements of K using a mass spectrometer instead of a manometer, one gains the significant advantage that, of the several gases which may be involved in complete pressure equilibration, data are taken only on the single gas with which one is concerned. However, 2 additional points must be

considered; instrument response time and gas phase mixing.

The rate of response of a manometer to an instantaneous pressure change is limited by manometer fluid viscosity and several other minor factors. In practice the Warburg manometer responds so fast to gas phase pressure change that, for nearly all ordinary uses, the inherent instrumental response lag is negligible. On the other hand, most commercially available mass spectrometers have gas inlet systems which offer major resistance to diffusion either on the high pressure side of the leak or in the low pressure region where molecular diffusion occurs. In the present adaptation of a Nier-Consolidated mass spectrometer these instrumental response lags have been minimized by reducing the diffusion barriers as much as was feasible by altering the design of the leak and gas inlet system to remove unnecessary constrictions. The speed of the mass spectrometer diffusion pump is high and the pump-out time of the mass spectrometer tube (time for half replacement of the gas in the ion source region) was in our case 2.5 seconds which is small compared with the half-time for equilibration across the gas-liquid interface in the reaction vessel.

The response of a mass spectrometer is somewhat slower than that of a manometer also because gas phase mixing is a much more important factor in the former. The most rapid response was observed at near vacuum pressures in the reaction vessel. Various background or carrier gases slowed down the response. The effect was a function of the carrier gas viscosity. As might be expected, the O_2 and CO_2 equilibration constants depended on the total pressure in the gas phase but not on the partial pressure of the gas being measured nor on the direction of diffusion (into or out of solution). Helium was chosen (in preference to nitrogen) as the carrier gas to be used in all experiments reported here. Measurements were carried out in a gas phase which was 96 to 98 % He and the remainder was a mixture of CO_2 and O_2, both isotopically enriched. The total pressure was approximately 1 atmosphere.

All experiments were run in the same rectangular Warburg-type reaction flask under essentially the same conditions except as regards light intensity. The volume of the cell suspension was 2 ml with a cell concentration of about 0.5 %. The shaking rate was 172 complete oscillations per minute at a vessel excursion of 17 mm. The value of the constant, K, in equation 4 was, for O_2 in this system, 0.06. This corresponds to a half-equilibration time of about 11 seconds.

As was anticipated above, knowledge of the K value characteristic of the system used in our experiments makes it possible to compute the ratio, M/M*, in solution from measurements of the ratio made in the gas phase. For a particular gas being measured the diffusion rate, R, across the interface is a linear function of the concentration difference through the barrier. The proportionality constant, referred to as the interface transfer constant, is k which is related to K by

$$K = k\left(\frac{a\,v + V}{v\,V}\right) \qquad (5)$$

where v and V are liquid and gas volumes respectively and a is the solubility coefficient. Therefore k is easily computed from K as determined experimentally and the concentration of dissolved gas, M_s, is given by

$$M_s = a\,M - \frac{R}{k} \qquad (6)$$

in which R is the steady state rate of gas transport across the interface and M is the concentration of the gas prevailing in the gas phase. The values of k for our experimental conditions were, for O_2, 0.13 and, for CO_2, 0.072 vol. units sec^{-1}.

Parenthetically, another method of evaluating k may be mentioned. If the density of the cell suspension is increased until R reaches a maximal value, it may be assumed that this condition prevails only when M_s in equation 6 becomes vanishingly small. Thus $k = R/aM$ approximately. It was noted that the 2 methods led to essentially the same value of k in spite of the fact that very different cell densities were employed. While this was the case for Ankistrodes-

TABLE I

SAMPLE DATA AND COMPUTATION RESULTS*

	EXPERIMENTAL PERIODS		
	DARK 45 MIN	LIGHT 29 MIN	DARK 31 MIN
R_{32}	−0.49	2.7	−0.68
M_{32}	143	278	318
R_{34}	−0.22	−0.04	−0.22
M_{34}	121	111	102.5
R_{44}	1.1	−1.2	1.3
M_{44}	115	125	127
R_{45}	0	−0.92	0.05
M_{45}	84.5	72	59.5
P_{O2}	−0.15**	5.7	0.3**
P_{CO2}	1.2	0.4	1.4
U_{O2}	0.6	3.1	1.3
U_{CO2}	0	2.8	0.2
ΔP_{CO2}		0.9	
ΔU_{CO2}		2.1	
$\Delta P_{CO2} + \Delta U_{O2} + U_{CO2}$		5.8	

* Expt. 2107. 5.3 μl cells. 26.0° C. Gas phase, CO_2 : O_2 : He (3 : 4 : 493). Constants (cf. equations 7 and 8) : j_{O2}, 0.932; F_{O2}, 36 × 10^{-6} per chart div.; f_{O2}, 6.36 × 10^{-6} ml (N.T.P.) O_2 sec^{-1} per chart div. min^{-1}; k_{O2}, 0.13 ml sec^{-1}; j_{CO2}, 1.121; F_{CO2}, 22.4 × 10^{-6} per chart div.; f_{CO2}, 5.17 × 10^{-6} ml (N.T.P.) CO_2 sec^{-1} per chart div. min^{-1}; k_{CO2}, 0.072 ml sec^{-1}. Units: M, chart div.; R, chart div. min^{-1}; all others, μml sec^{-1} μl^{-1}.

** Oxygen production may be assumed not to occur in darkness; departure of tabular values from zero indicates experimental error.

mus in phosphate buffer, it may have been fortuitous. Other experimental material or different media may influence interfacial properties in such a way that k becomes a significant function of cell concentration. We found this to be the case with another organism (Ochromonas). Complications of this kind also were reported by Habermann for chloroplast suspensions (11), and were noted by Dr. Robert Emerson for Chlorella suspensions (personal communication).

For a given kind of gas we were concerned in these experiments with 2 isotopic species; these must be considered separately. A pair of M_s values were calculated from measurements of M and R for the respective isotopic masses. Thus a pair of isotope concentrations—consequently a ratio—in the liquid phase was determined. Ratios calculated in this way, being much better approximations of the ratios existing at the site of gas consumption, were used in preference to the gas phase ratios as measured. From these considerations it is possible to improve on equations 2 and 3, which were first approximations involving only gas phase quantities, by substitution of corresponding expressions based on liquid phase quantities obtained by taking into account the diffusion barrier at the gas-liquid interface. Corrections of this sort also were applied to mass spectrometer data by Horwitz and Allen (12).

Values of M and M* and of their rates of change were measured in the gas phase in arbitrary units of pen excursion on the recorder chart. For this and for other reasons several conversion factors must be introduced in order that results may be expressed in the desired units. The overall calculations are indicated by the following equations which are revisions of equations 2 and 3:

$$P = (Rfj) - (R^*fj)\left[\frac{a\,MF - Rf\left(\frac{1}{k}\right)}{a\,M^*F - R^*f\left(\frac{1}{k}\right)}\right] \quad (7)$$

$$U = R^*fj\left[1 + \frac{a\,MF - Rf\left(\frac{1}{k}\right)}{a\,M^*F - R^*f\left(\frac{1}{k}\right)}\right] \quad (8)$$

for which the following symbols are defined (for the case where O_2 is the gas considered):

P and U = production and consumption rates for O_2. Units, ml per sec per μl cells $\times 10^{-6}$.

M and M* = concentrations of the 2 isotopic forms of O_2 measured on the recorder chart as mass spectrometer peak heights for the respective masses. Units, arbitrary scale divisions.

R and R* = rates of change of M and M* respectively. Units, scale divisions per minute.

$j = \left[\frac{a\,v + V}{Vv}\right]\left[\frac{273}{T}\right]$, where v and V are liquid and gas volumes (ml) and T is the absolute temperature.

F = C/S, where C is the oxygen fraction of the gas used for calibration and S is the mass spectrometer peak height (in scale div) for mass 32 when measuring the calibration gas at 1 atmosphere.

$f = F \cdot V/60$.

k = interface transfer constant as previously defined. For analogous measurements on CO_2, masses 44 and 45, a set of similar equations with comparable constants was applied. The units of P_{O2}, U_{O2}, P_{CO2}, and U_{CO2} are ml sec^{-1}. The final values are expressed on a unit cell volume basis.

Results and Discussion

Figure 4 shows an example of mass spectrometer data for the metabolism of 2 isotopic forms of O_2 (masses 32 and 34) and 2 of CO_2 (masses 44 and 45). The essential measurements on a chart record such as figure 4 are recorded in table I along with results of computations leading to the true production and consumption rates of both O_2 and CO_2. The table also shows the final result of light-dark comparisons. Note that the O_2 production rate in the light period, Po_2 is nearly the same as the sum of: deficit in CO_2 production, enhanced O_2 consumption, and CO_2 uptake as shown by the last entry in the table. This agreement is predicted by equation 1. Data on 13 light intervals, such as that on which table I was based, with dark periods before and after for comparison, were obtained over a range of light intensities. Values of U_{CO2}, ΔP_{CO2}, and ΔU_{O2} calculated for each illumination period, were plotted against corresponding values of P_{O2} in figures 5 to 7.

Figure 5 shows the relation between rate of CO_2 utilization and O_2 production. If the photosynthetic quotient were actually unity, the points would all lie along the diagonal. This was not the case as the uptake of CO_2 fell short of being equivalent to O_2 production especially at higher rates of photosynthesis.

Figure 6 shows the light induced deficit in respiratory CO_2 production. Compared with the dark rate CO_2 production was reduced to about one half by light, an effect which varied little with photosynthetic rate.

Figure 7 shows the photostimulated fraction of the O_2 consumption which was negligible at low light intensities but which became large at higher rates of O_2 evolution. It may be noted that, at low light intensities, neither stimulation nor inhibition of respiratory O_2 uptake occurred. Under these conditions reliable measurements of photosynthetic O_2 production could have been made on such Ankistrodesmus suspensions by ordinary methods not involving the use of tracers. However, in the case of CO_2 metabolism, results were less reassuring. Because of the suppression of CO_2 production in light of even low intensity, measurements without tracer of net CO_2 exchange are not readily interpreted.

Each point in figure 8 is the absolute sum of ordinate values in figures 5 through 7 plotted against O_2 production rate. These points lie along the diagonal indicating that, over the range of light conditions employed, the rate of production of photosynthetic reductant (corresponding to the rate of oxidant production and measured as O_2 evolution) was equivalent to the sum of the CO_2 consumption rate, the deficit in the rate of CO_2 production, and the increase in the rate of O_2 absorption. This is the condition imposed by equation 1. Thus, figure 8 demonstrates that the data were generally in agreement with the equation over a relatively wide range of light intensities (up to about 6 times compensation). The data are consistent with the assumption of an effect of light on respiratory metabolism mediated by a reductant rather

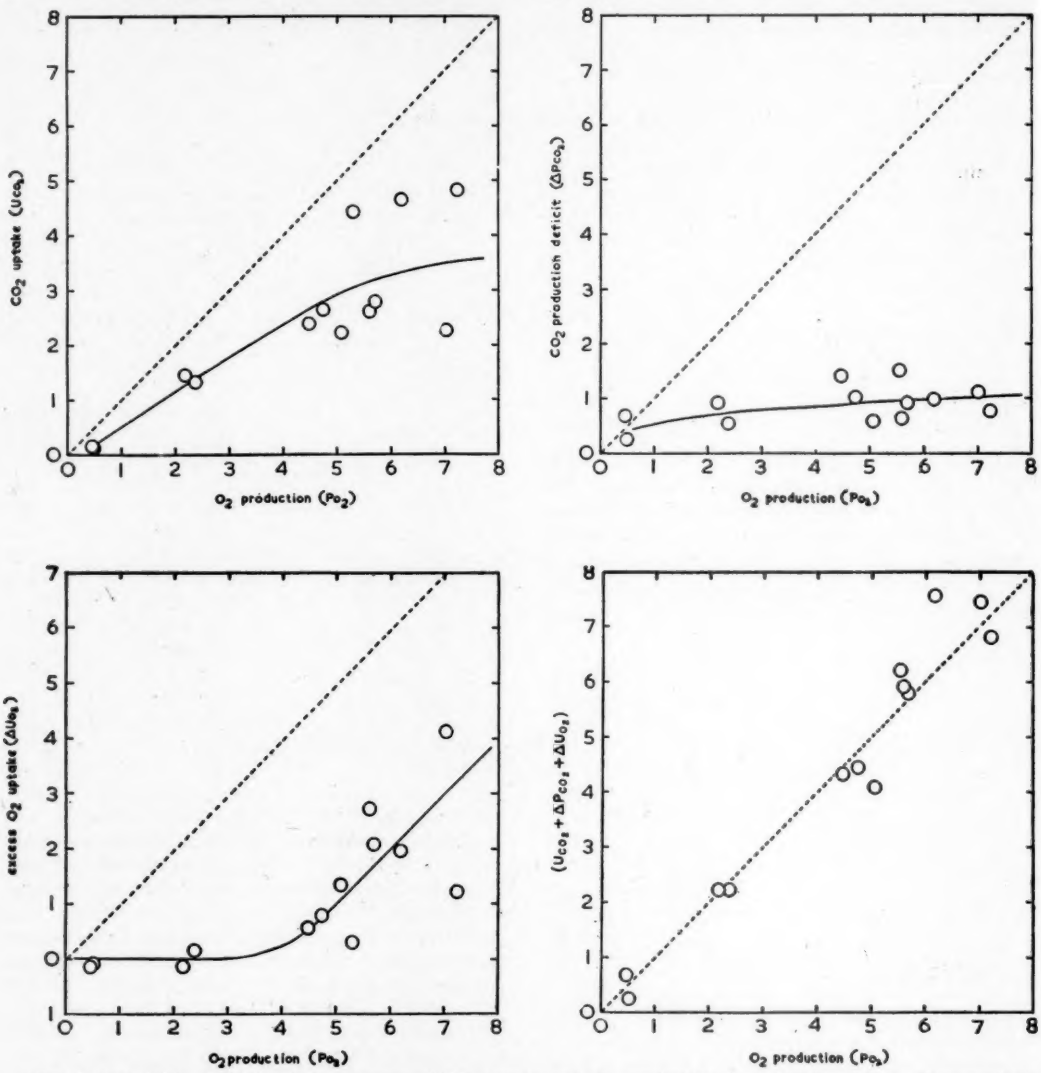

FIG. 5 (*top, left*). Relation between rates of CO_2 uptake and O_2 production in the light. If the photosynthetic quotient actually were unity, all points would lie on the diagonal.

FIG. 6 (*top, right*). Relation between deficit in CO_2 production rate caused by light and the rate of O_2 production.

FIG. 7 (*below, left*). Relation between the enhancement by light of the rate of O_2 consumption and the O_2 production rate.

FIG. 8 (*below, right*). Comparison of light induced deficit of CO_2 evolution rate, increment in O_2 consumption rate, and CO_2 uptake rate with the rate of O_2 production. Prediction according to equation 1 requires that the points should lie along the diagonal.

than by an oxidant. At low light intensities—low rates of reductant generation—only CO_2 evolution is affected. At higher light intensities enhancement of O_2 consumption occurs as well.

From kinetic data of this type it is of course not possible to decide where in the oxidative process the photosynthetic reductant interacts with respiratory metabolites, coenzymes, or electron carriers. It may be noted also that the enhanced O_2 consumption observed at the higher light intensities is not readily dismissed as photooxidation not directly related to photosynthesis and respiration, since the increment in O_2 consumption was matched by increasing O_2 production. If the usual kind of photooxidation were superimposed on photosynthetic and respiratory gas exchange, results would not agree stoichiometrically with equation 1.

During some of the experiments described above further data were taken while the cell suspensions were allowed to photosynthesize until the CO_2 was depleted to the level of CO_2-compensation. As this condition was approached, the CO_2 isotope ratio, M_{44}/M_{45}, became so large that calculations of production and consumption rates were subject to large error as the net rate of CO_2 uptake approached zero. Oxygen computations were as reliable as in other experiments. Figure 9 illustrates the changes which were observed in 2 representative experiments. After CO_2-compensation was attained, the rate of O_2 consumption usually was higher and O_2 production invariably lower than was the case for normal photosynthesis with an adequate concentration of CO_2. Evolution and uptake of O_2 were then steady and essentially in balance. Just before the condition of compensation was reached, a transient stimulation of O_2 exchange sometimes was observed (as in experiment 1707 of fig 9). When such experiments were performed on suspensions without supplementary glucose, this transient usually was especially pronounced.

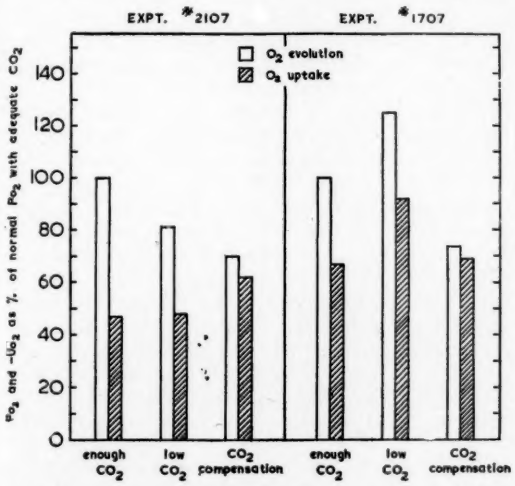

FIG. 9. Comparison of rates of O_2 production and consumption under conditions of CO_2 depletion. Two separate experiments are shown.

It is reasonable to suggest that results such as these, obtained as the cells run out of CO_2, demonstrate a change in fate of the photochemical reductant which, when deprived of its normal oxidant, reduces O_2 instead. The steady state which finally obtains is one in which O_2 consumption is elevated sufficiently to match—or nearly match—O_2 evolution; i.e., net gas exchange approaches zero and an O_2 exchange in ratio 1 : 1 prevails. The rates of O_2 consumption observed under such compensation conditions, while higher than normal for the light intensity used, were about equivalent to—but did not exceed—the maximal rate of extra O_2 uptake observed in any light period where sufficient CO_2 was present. This maximal rate was observed only at a significantly higher light intensity in the case of normal photosynthesis. From the fact that O_2 production rates were not maintained under CO_2 deficient conditions, as illustrated in figure 9, we may deduce that O_2 reduction by photochemical reductant either is less efficient than CO_2 reduction or, more probably, when CO_2 compensation prevails, secondary changes may inhibit energy transfer from chlorophyll, increase the probability of back reactions, and thus lower the O_2 production rate as suggested by Franck and coworkers (7, 8, 9, 17).

SUMMARY

The gaseous metabolism of the alga, *Ankistrodesmus braunii*, was measured in darkness and in light of various intensities. Cells were suspended in phosphate buffer containing glucose and the gas phase, enriched with O_2 and CO_2 isotopes, was monitored with a recording mass spectrometer. In contrast to earlier results with other experimental material, isotope exchange involving CO_2 was not prohibitively rapid so that with this gas as well as with O_2 it was possible to calculate simultaneous production and consumption during the light. These calculations depended on knowledge of the isotope ratio prevailing at the site of gas consumption and, since the ratio (especially in the case of O_2) generally was not the same in liquid and gas phases, corrections were introduced which took into account the effect of the diffusion barrier at the gas-liquid interface; calculations therefore were based on isotope ratios prevailing in the liquid phase which were considered close approximations to those at the intracellular reaction site. It was found that light induced an inhibition of CO_2 evolution which was nearly independent of light intensity whereas O_2 consumption in the light was unaffected at low intensity but enhanced at high intensities. The results were quantitatively in agreement with interaction between a photosynthetic reductant and the respiratory mechanism.

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A pure culture of the experimental organism was kindly supplied and identified by Dr. R. E. Norris.

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KINETIC RELATIONSHIPS BETWEEN PHOTOSYNTHESIS AND RESPIRATION IN THE ALGAL FLAGELLATE, OCHROMONAS MALHAMENSIS[1]

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Studies on the influence of light on respiration have yielded different results with different organisms. In some instances results were interpreted as evidence for light stimulation of respiration (3, 4, 5, 6), in other cases for photoinhibition (8, 13), and in still other examples for a negligible effect (1). With a given species simultaneous measurements of respiratory and photosynthetic O_2 metabolism have shown that the influence of light on respiratory processes varied with experimental conditions (3, 4). The fact that conditions have not always been strictly comparable in different investigations probably accounts for some contradictory results.

In the preceding paper, which also dealt with the problem of accounting for manifold influences of light —or photosynthetic metabolism—on respiratory processes, a very simple model was proposed (figure 1, page 226. In that model photosynthesis is considered as an oxidation-reduction reaction yielding oxidant and reductant at equal rates. The oxidant is the precursor of molecular O_2; the reductant serves ultimately to reduce CO_2. It is assumed that the reductant, but not the oxidant, may react also with components of the respiratory mechanism. Such interaction could result in either an increased O_2 consumption rate, a decreased CO_2 production rate, or both. Interaction of some of the reductant with respiratory intermediates would result in diversion of this amount of reductant from its photosynthetic role of CO_2 assimilation.

The photosynthetic-respiratory interactions proposed in the model have several specific consequences which should be experimentally observable. The respiratory quotient ($+CO_2/-O_2$) would be decreased and the photosynthetic quotient ($+O_2/-CO_2$) would be increased. The rate of photosynthetic O_2 production would be unaffected whether or not the postulated interactions occur, since by assumption the photosynthetic oxidant is not involved. A further consequence of the model is the stoichiometric equivalence of O_2 produced in light to the sum: CO_2 consumed + light-induced extra O_2 uptake + light-induced deficit in respiratory CO_2 production.

Perhaps only because of its simplicity, this model was able to explain quantitatively some light effects on respiration in an arbitrarily selected organism, Ankistrodesmus (4). The present paper describes similar experiments on the physiologically heterodox genus, Ochromonas.

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MATERIALS AND METHODS

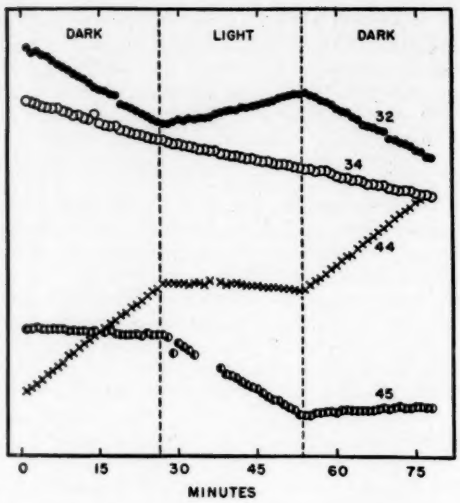
The Chrysophyte flagellate, *Ochromonas malhamensis* isolated by Chen and described by Pringsheim (10), was used in these experiments. A defined medium allowing physiological experimentation was devised by Hutner, Provasoli and Filfus (7) and a number of physiological studies have since been carried out. The role of photosynthesis in the metabolism of the organism has been studied by Myers and Graham (9) and by Weis (14) and dark metabolism was studied by Reazin (11). The ability of this flagellate to carry out photoreduction was investigated by Vishniac and Reazin (12). An investigation of the effect of culture conditions on the development of enzymes required for CO_2 reduction was carried out by Reazin and Fuller (personal communication).

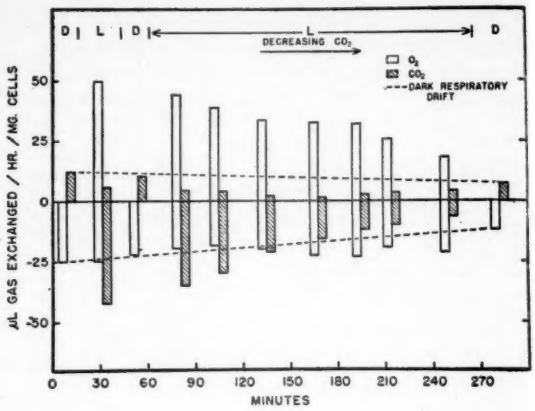
A characteristic of Ochromonas, advantageous for the present work, is its relatively rapid rate of respiration and low maximal rate of photosynthesis. In past experiments from this laboratory rather low rates of gas exchange (i.e., relatively low light intensities) were employed for technical reasons. Reliable results were confined to the lower (nearly linear) portion of the light intensity—photosynthetic rate curve. With Ochromonas, even at saturating light intensities, accurate measurements of both respiration and photosynthesis were possible.

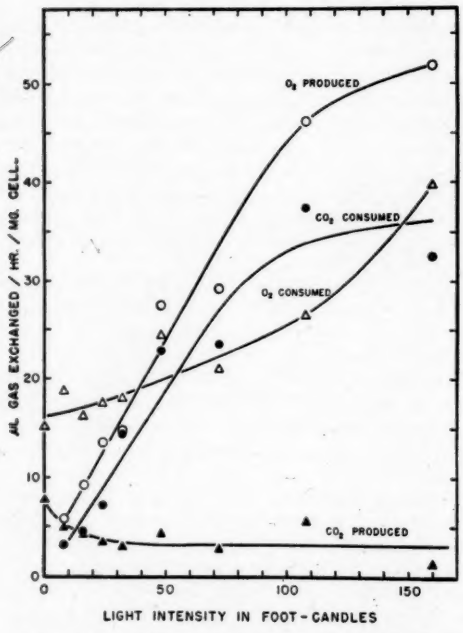
Another feature of Ochromonas which proved useful was the ease with which its respiratory rate could be reduced by starvation and subsequently enhanced by exogenous substrate. This permitted greater flexibility in the design of experiments to examine the kinetic interrelations between photosynthetic and respiratory metabolism, since rates of both processes were subject to experimental control over a considerable range.

Cells were cultured at 23° C in a defined medium (7) modified by the substitution of ammonium sulfate for ammonium citrate and calcium chloride for calcium carbonate. Light intensity was maintained at 25 ft-c supplied by fluorescent tubes and filtered through orange glass (Corning no. 348). Cultures containing 30 ml in 125 ml cotton stoppered Erlenmeyer flasks were aerated by shaking once daily. More vigorous aeration and higher light intensity were avoided because of evidence that such treatment prevents complete development of the photosynthetic apparatus (14).

After 4 to 5 days growth, cells were harvested by centrifugation at 500 × G: they were washed once with a solution containing the major minerals of the culture medium; finally they were resuspended in







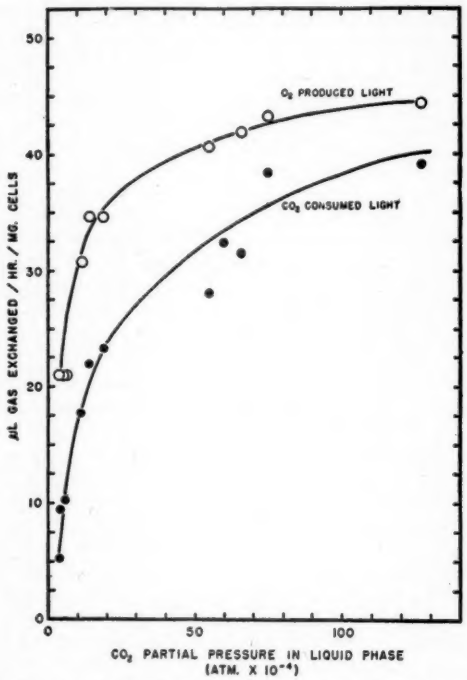


FIG. 1 (*top, left*). Example of mass spectrometric data for 2 isotopic forms of CO_2 (mass 44 and 45) and 2 of O_2 (mass 32 and 34). Ordinate: relative partial pressures of gas.

FIG. 2 (*bottom, left*). The effect of light intensity on gas exchanges by starved cells. Gas phase, $CO_2 : O_2 :$ He (2 : 3 : 95). Cells starved 24 hours.

FIG. 3 (*top, right*). The effect of decreasing concentration of CO_2 on gas exchanges by starved cells in the light. Red light from 250-watt tungsten lamp filtered through Corning no. 2403 red glass filter. Initial gas phase: $CO_2 : O_2$: He (1 : 3 : 96). Cells starved 18 hours.

FIG. 4 (*bottom, right*). The effect of CO_2 partial pressure on O_2 production and CO_2 consumption by starved cells in the light. Experimental conditions as in figure 3.

0.02 M phosphate buffer (pH 5.5) and either used directly or starved prior to measurements of gas exchange. Starvation was carried out in the dark at 23° C, sterility being maintained throughout the starvation period.

All experiments were carried out in a rectangular reaction flask attached to the gas inlet system of a mass spectrometer. The bath in which the flask was immersed was at 28° C. The adaptation of the mass spectrometer for use in such experiments has been described (1) and the spectrometer leak housing to which the vessel was attached has been illustrated (8).

Light was supplied from a 250-watt tungsten filament projector lamp through appropriate columnating lenses and was introduced into the constant temperature bath to illuminate the reaction flask as described previously (2). Red light was obtained by placing a glass filter (Corning no. 2403) in the bath between the light source and the reaction flask.

In those experiments in which data on metabolic exchanges of CO_2 were sought, CO_2 enriched with respect to mass 45 ($C^{13}O_2$) was used. The isotopic O_2 was enriched with mass 34 ($O^{16}O^{18}$).

Dry weight was used as a measure of cell material.

To calculate rate of gas exchange in tracer experiments it was necessary to correct for diffusion lag across the liquid-gas interface. The reason for this correction and the manner of making it was explained in the preceding paper (4).

At the beginning of an experiment the experimental suspension with appropriate addenda was pipetted into the reaction flask and the latter, attached to the mass spectrometer gas inlet assembly, was placed on a shaking device in the constant temperature bath. Isotopically enriched CO_2 and O_2 were introduced into the gas phase which was primarily He. Data were recorded continuously on the several isotopic forms of the metabolic gases. Computations of production and consumption rates of O_2 and CO_2 were made from results such as those of figure 1. In general the experimental procedures and methods of handling the data were essentially the same as have been described previously (4).

Results and Discussion

The metabolic relationships of interest are the effects on rates of CO_2 production and O_2 consumption in the light brought about by starvation, by changes of light intensity, and by altering concentrations of O_2 and CO_2 in the milieu.

Starvation: After starvation in the dark the endogenous respiratory rate was reduced. Addition of glucose enhanced the O_2 consumption rate of starved cells up to the same level as that of unstarved cells. The respiratory rate of unstarved cells was found to be relatively insensitive to illumination whereas the rate of O_2 uptake by starved cells was nearly doubled by saturing light intensities. The light effect was reversible. These light relations are illustrated by the example given in table I. The data in the table were computed from experiments in which tracer O_2 was employed. Thus these data represent actual O_2 consumption rather than net O_2 change.

With respect to CO_2 production, both starved and unstarved cells were observed to be light sensitive. A significant reduction in rate of respiratory output of CO_2 was induced by light in both cases. The 1st line in table II and figure 2 furnish examples of these effects. These data and all data to follow were taken from experiments in which both tagged O_2 and tagged CO_2 were used and thus represent total rather than merely net rates.

We may think of respiration in terms of a flow of "substrate electrons" toward O_2. Starvation may be considered to deplete the supply of endogenous respiratory substrate thus reducing the rate of electron transport. Should photosynthetic reductant compete with substrate electrons, such competition would be observed as a light induced deficit in the rate of CO_2 evolution. This evidence of competition was found with both starved and unstarved cells (table II). If, on the other hand, photosynthetic reductant only results in an increased rate of electron transport to O_2, no effect on CO_2 production would be expected; only the rate of O_2 utilization would be enhanced. This latter effect was observed with starved cells in which the electron transport system presumably was not functioning at maximal capacity. Since, with unstarved cells, a light induced change in O_2 consumption was not observed, it may be suggested that the electron transport system already was operating at full capacity in the dark; addition of further reductant (of photochemical origin) could not produce an increase pro-

Table I

Effects of Starvation and Light on the Rate of Oxygen Consumption of Ochromonas*

Successive periods	Unstarved cells**	Starved cells**
Dark	35	15
Light	33	28
Dark	34	17

* Starvation period, 24 hours dark. Gas phase, $O_2 : CO_2 : He$ (2 : 2 : 96).
** Units, μl O_2 consumed per mg dry wt per hour.

Table II

Effects of Light on Gas Exchanges of Ochromonas *

	Unstarved cells**	Starved cells**
Deficit in CO_2 evolution	16	7
Enhancement of O_2 uptake	— 1	8
CO_2 consumption	23	27
	38	42
O_2 production	36	45

* Same conditions as for table I.
** Units, μl mg^{-1} hr^{-1}.

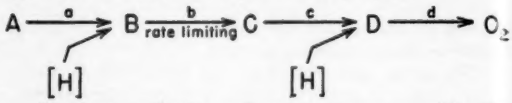
vided the point of influence lay below (with respect to O_2) whatever redox reaction was rate limiting. A direct photochemical reduction of O_2 itself thus seemed improbable in the case of Ochromonas, as this could occur regardless of the degree of saturation of the electron transport mechanism by substrate electrons.

LIGHT INTENSITY: Using starved cells, since photo-effects on respiration were found to be optimal in them, the production and consumption rates of O_2 and CO_2 were studied over a light intensity range from darkness to light saturation (fig 3). Production of O_2 and uptake of CO_2 proceeded more or less linearly with light intensity below saturation. Respiratory CO_2 production was affected at even the lowest light intensities and an inhibition in the neighborhood of 30 to 50 % was established even at a light intensity approximating O_2 compensation. Above this relatively low light intensity no further reduction of CO_2 evolution occurred.

A very different pattern was found for O_2 consumption. At light intensities up to about compensation for O_2, light failed to exert a marked effect. Above that compensation point, an increasing photostimulation of O_2 uptake rate was observed. This acceleration approached 100 % at light saturation and was then about equivalent to the maximal stimulation of O_2 uptake rate which was observed when glucose was supplied to starved cells.

Over the entire range of light intensities the photosynthetic quotient was somewhat above unity although excess of O_2 production over CO_2 consumption became more pronounced only at the higher intensities. That even slight photosynthetic activity depressed the CO_2 evolution rate implies that light generated reductant can compete very favorably with substrate electrons, maintaining nearly the same overall rate of O_2 reduction with photochemical reductant in lieu of substrate electrons. A maximal (ca. 50 %) photoinhibition of CO_2 production was achieved by rather little light and was not increased by several-fold further increase of intensity. This suggests a dual source of respiratory CO_2. Such behavior would be expected of a system having 2 mechanisms for CO_2 evolution with approximately equal functioning capacity, if one is directly accessible to a depressing influence of photosynthetic reactions and the other is not. Respiratory CO_2 production by 2 chemically distinct pathways may be involved. Alternatively intra- and extra-chloroplastic systems of CO_2 evolutions may operate in Ochromonas.

The fact that light of low intensity had little effect on the O_2 consumption rate yet, at higher intensity, considerable stimulation occurred can be interpreted as evidence for 2 types of interaction between the photosynthetic reductant and the respiratory process. For the light generated reductant to affect the rate of O_2 consumption it must react, if not with O_2 directly (which was considered improbable), then with a member of the coenzyme-carriers-oxidase system at some point beyond (on the way toward O_2) the rate limiting reaction step in the overall redox sequence (b in the example below, where components of the electron transport system are referred to only by symbols since they remain unidentified in Ochromonas. Photosynthetic reductant is represented by [H]).



If photosynthetic reductant competes with the transport of substrate electrons at reaction a, depression of CO_2 evolution would occur without the rate of O_2 uptake being enhanced, because the point of competition is below the rate limiting step. This is characteristic of the effects observed at low light intensity where the predominating influence is on CO_2 production. If photosynthetic reductant competes with substrate electrons at some point beyond b, the later stages of the transport sequence could react still more rapidly and an increased rate of O_2 uptake would result. This is characteristic of the effects observed at the higher light intensities.

The above suggestion provides a unified explanation for 2 apparently diverse effects of light on Ochromonas respiration. At both high and low light intensities the postulated influence is one of competition between substrate electrons and photosynthetic reductant for components of the respiratory electron transport system. The different manifestations of this competition at different light intensities arise because the predominant influence is in one case below—in the other case also above—the rate limiting redox reaction. This second point of interaction comes into play only at high light intensity.

PHOTOSYNTHETIC QUOTIENT: With increasing light intensity the quotient, $+O_2/-CO_2$, was found always to increase. Since the rate of photochemical reductant generation increases with increasing light intensity, correlation between increased photosynthetic quotient and higher light intensity suggests that the CO_2 is in some kind of indirect competition with an alternative oxidant such as the respiratory electron transport system. By depleting CO_2, a greater fraction of the photochemical reductant should be accounted for not in photosynthesis (CO_2 utilization) but by the alternative fate of reducing components of the respiratory system. Figure 3 shows the results from an experiment in which CO_2 tension in the experimental vessel was gradually depleted by photosynthesizing Ochromonas cells. Respiratory drift was apparent as the dark O_2 uptake declined with time (onset of starvation). However, the rate of O_2 consumption in the light did not change significantly. Therefore a light stimulation of respiration (effect of photochemical reductant reducing the electron transport system rather than CO_2) was observed and was greater the lower the CO_2 tension.

A series of experiments were carried out in order to demonstrate in a more definitive manner the competition between the oxidants, CO_2 and O_2. It was found, unexpectedly, that from 1 to 2 % CO_2 was re-

quired in order that photosynthesis in starved Ochromonas cells proceed without CO_2 limitation. A quotient, $+O_2/-CO_2$, of about 1.1 usually was observed. As shown in figure 4, at 10×10^{-3} atmospheres dissolved CO_2 the quotient was 1.2 to 1.4; at 2×10^{-3} atmospheres it was 1.6; at 1×10^{-3} atmospheres it rose to 4.2. For technical reasons the data taken at lower CO_2 tensions are less reliable, but an obvious trend is revealed in figure 4. The lower the concentration of CO_2, the higher the photosynthetic quotient. A greater fraction of photosynthetic reductant is not involved in CO_2 assimilation but reacts with the respiratory system to enhance O_2 consumption or to depress CO_2 production in accordance with the model employed. In this sense a competition is revealed between CO_2 and the respiratory system of Ochromonas.

It was noted earlier that the model used here to explain the several interactions between light generated reductant and respiration demands, under all conditions in the light, that O_2 production rate, P_{O_2}, should be equal to the sum : deficit in rate of CO_2 evolution, ΔP_{CO_2}, enhancement of O_2 uptake rate, ΔU_{O_2}, and the rate of CO_2 utilization, U_{CO_2}.

$$\Delta P_{CO_2} + \Delta U_{O_2} + U_{CO_2} = P_{O_2} \qquad (1)$$

Throughout this study where the above 4 quantities were determined, equation 1 was found valid within experimental error. Examples of the equivalence are noted in table II.

The readiness with which reductant of photochemical origin exerts an influence could be an immediate result of respiratory production and photosynthetic consumption of CO_2 being mediated by the same enzyme system or by bound enzymes in close juxtaposition.

Summary

Gas exchanges of *Ochromonas malhamensis* were studied in dark and in light using a recording mass spectrometer to analyze the partial pressure changes of isotopically enriched CO_2 and O_2 within the experimental vessel. Simultaneous production and consumption rates of both CO_2 and O_2 were determined. Light intensity, partial pressure of CO_2, and state of nutrition of the cells were varied. At light intensities below compensation, illumination had slight influence on rate of O_2 consumption; at higher intensities uptake was stimulated. CO_2 production was inhibited even at very dim light but with increasing intensity no further depression of CO_2 production rate occurred. Light had a more pronounced effect on respiration in starved cells than in cells with ample endogenous substrate. Quantitatively the behavior of Ochromonas was consistent with a model which accounts for an influence of light on respiration mediated by a photochemically generated reductant. At low light the O_2 consumption rate was maintained while the photosynthetic reductant competes with reductant of respiratory origin. At higher light a second type of interaction enhancing the O_2 uptake rate, was superimposed on the first effect.

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Some of the work reported here was taken from a thesis presented by one of us (D.W.) in partial fulfillment of requirements of the Ph. D. degree, Yale University, 1955.

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PYOCYANINE AND PHOSPHORYLATION WITH CHLOROPLASTS[1, 2]

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The process of formation of oxygen by illuminated chloroplast preparations in presence of added H-acceptors resembles the converse of the process of the oxidation of H-donors by oxygen with the Keilin-Hartree muscle preparation derived from mitochondria (9). In the former process the H-transport is against the chemical potential owing to transformation of the light energy. The discoveries of the photosynthetic phosphorylation with chloroplast preparations by Arnon, Whatley and Allen (2) and with the chromatophores from a photosynthetic bacterium by Frenkel (4) were of supreme significance in the development of the biochemical study of photosynthesis. Furthermore they indicated a similarity of the chloroplast to the mitochondria which was already partly apparent from the comparative study of cytochrome components in the two subcellular structures (10, 11). In oxidative phosphorylation with mitochondria the H-transport is dependent upon the presence of both inorganic phosphate and an acceptor for the phosphate group. A similar condition has been shown to operate in the chloroplast by Arnon, Whatley and Allen (6). The reduction of an H-acceptor and concurrent O_2 production by the illuminated chloroplast system is to a very significant extent coupled with phosphorylation. Moreover the phosphate acceptor—adenosine diphosphate (ADP)—is the same for the chloroplasts and the mitochondria. With mitochondria the coupling of H-transport and phosphorylation can be removed either by the presence of certain reagents or by the treatment used for the Keilin-Hartree preparation. In such a case active H-transport is shown without any concurrent phosphorylation. An analogous process of uncoupled H-transport is shown by the chloroplast preparations (4, 16, 17) either by addition of reagents or by a physical treatment. The important difference between chloroplasts and mitochondria apparently is in the direction of hydrogen transport and it might be legitimate to term the phosphorylation with chloroplasts as "reductive" in contrast to the oxidative phosphorylation with mitochondria. It has been shown that phosphorylation can occur with chloroplasts in light without any O_2 production and without the corresponding stoichiometric reduction of any H-acceptor. Thus a situation, named by Arnon "cyclic phosphorylation," would seem to involve a reduced product being constantly oxidized not by molecular oxygen but by a possible photochemically produced precursor of this gas. In the earlier experiments of Arnon and Whatley O_2 appeared to be necessary. In this case, presumably, a reduced product was being reoxidized by molecular oxygen. Thus in order to obtain the phosphorylation reaction with chloroplast preparations one essential condition must be to establish the photochemically driven H-transport. This condition can be met by adding certain "cofactors" to the washed chloroplast preparations.

The present investigation was undertaken in order to see how specific the two reagents used by Arnon (K_3 and FMN) were in relation to the phosphorylation reaction; and to see if the activity could be related to reactions of chloroplast with H-acceptors. A preliminary report of this work was given at a meeting of the Biochemical Society. (15).

MATERIALS AND METHODS

PLANT MATERIAL: During the period January 14 to April 14, 1957, sugar beet leaves from greenhouse material were made available to us through the kindness of Dr. G. D. H. Bell and the staff of the Plant Breeding Station. Also we are grateful to Dr. J. Swabey, Research Station of Plant Protection Ltd., for a batch of spinach plants. During the period July 1 to 21 of the same year leaves of spinach beet (Swiss chard) were available from plants sown in the open near Cambridge in May. The plants were used when possible at a stage between 8 to 12 leaves. The leaves used were those which seemed to have reached a point slightly less than their full size and when they could be distinguished by their minimum resistance to crushing. In the Swiss chard leaves used no starch could be detected but when the leaf extracts were centrifuged at low speeds a white deposit consisting of calcium oxalate crystals was always obtained.

REAGENTS: Phenazine methosulphate was made from phenazine by dissolving in hot xylene (dried over K_2CO_3) 15 parts, adding slightly more than 1 equivalent of freshly redistilled dimethylsulphate and heating to 115 to 120° C until the maximum yellow precipitate was obtained (about 30 mins). The mixture was cooled, the solid filtered off and washed with dry ether, followed by a very little absolute alcohol.

As far as possible, all contact with moist air was avoided. The crude product was recrystallized from alcohol with the addition of ether. Pyocyanine was prepared according to McIlwain (13) and purified by repeated passage from chloroform to acid, and isolated as the crystalline free pig-

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ment. The preparation was kept at −15° C. Resorcin blue or Lacmoid was purified by dissolving at pH 7.5 and salting out with the addition of NaCl. The following abbreviations are used: Adenosine diphosphate, ADP; Adenosine triphosphate, ATP; Flavine nucleotide, FMN; 2-methyl-1,4-naphthoquinone, K_3.

CHLOROPLASTS: Chloroplasts were prepared by a method similar to that of Arnon (4) in a buffered solution containing 0.35 M NaCl, 0.001 M $MgCl_2$, and M/15 2-amino-2-hydroxymethylpropane-1,3-diol (TRIS) at pH 7.4. For some experiments the $MgCl_2$ was omitted and added to the reaction mixtures as required. Washed leaves (25 to 30 g) from which the midribs had been removed were illuminated in tap water for about 1 hour. They were then shaken dry, shredded into a mortar and rapidly pulped in 50 to 60 ml of buffer. The pulp was squeezed in a double thickness of muslin and the expressed juice centrifuged at low speed (average 500 G) for 1 minute. The supernatant was retained and centrifuged again (1000 G) for 10 minutes. The 2nd supernatant was discarded and the chloroplasts uniformly resuspended in about 10 ml of buffer. This suspension was brought to about 60 ml by the addition of more buffer and then centrifuged for an additional 10 minutes (1000 G). The supernatant was again discarded and the chloroplast pellets resuspended as before but using either buffer or distilled water to give a final volume of 10 ml. All the apparatus and the solutions used were kept at about 0° C throughout.

Microscopic examination showed that the final suspensions consisted largely of intact chloroplasts. In addition there were some smaller green fragments and a few very small particles which may have been mitochondria. When distilled water, rather than the buffer solution, had been used as the suspending medium the chloroplasts appeared to be less definite in outline and soon disintegrated.

Reactions were carried out in 1.5 × 10 cm glass centrifuge tubes with rounded ends. Each tube contained 0.1 ml of chloroplast suspension (about 0.05 mg chlorophyll), 0.1 ml of TRIS-NaCl buffer at pH 7.4, 1 μM ascorbate together with ADP, orthophosphate and other reactants as specified, in a final volume of 0.3 ml. A few seconds of centrifugation at low speed ensured that any small drops of added reactants remaining on the sides of the tubes were drawn to the ends. In the winter and early spring 1.5 μM of orthophosphate was used in each tube. In the summer this was increased to 2.5 μM of ADP and 2.0 μM of phosphate to facilitate measurements with more active chloroplast preparations.

In experiments designed to compare the activity of "broken" chloroplasts (resuspended in distilled water) with "whole" chloroplasts (resuspended in TRIS-NaCl buffer) additional buffer was added to reaction mixtures containing the "broken" chloroplasts in order that the final concentrations of TRIS-NaCl were the same in each case. In all cases tubes were chilled before the addition of the chloroplasts.

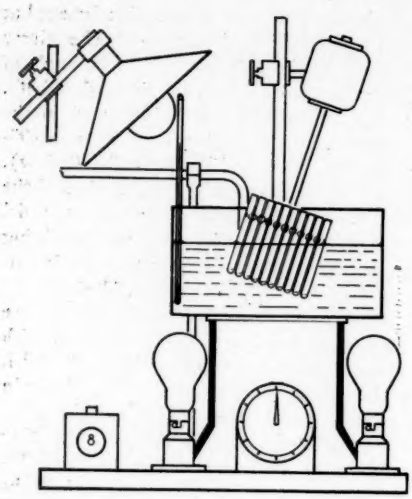
ILLUMINATION. Eighteen tubes were attached with "terry" clips to the rim of a cork disk fastened at its center to a motor driven stirring rod. The stirrer was adjusted so that the disk was held at an angle of about 20° to the surface of the water in a glass bath. The lower ends of the tubes were immersed in the water in the bath which was illuminated by one 200-watt bulb (above) and four 150-watt bulbs (below). When the stirring rod was rotated the motion of the inclined disk caused the contents of the tubes to swirl round in a thin film facilitating light saturation, mixing and temperature maintenance (see fig 1). Dark controls were carried out in tubes painted black.

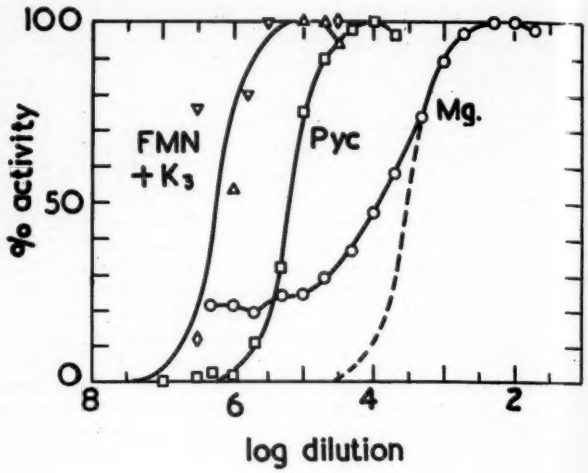
For some anaerobic experiments the tubes were evacuated prior to illumination. This was done with a mercury pump attached to a hypodermic needle which was inserted for 90 seconds through a rubber bung in the mouth of each tube. The contents of the tubes were vigorously agitated during evacuation. In other experiments the reaction mixtures were increased 10-fold and illuminated in closed 50 ml conical flasks through which oxygen-free nitrogen was passed for 10 minutes before illumination. In these experiments inorganic phosphate was determined in an 0.3 ml sample of the reaction mixture.

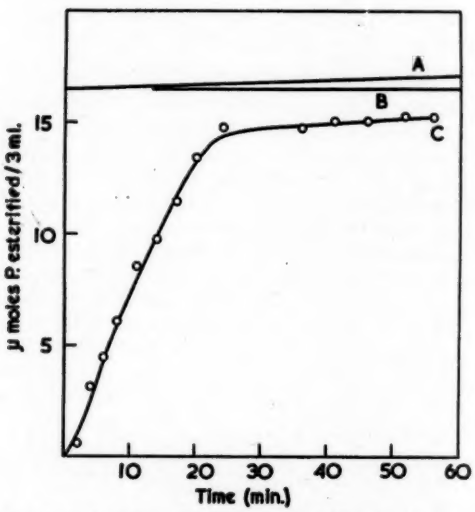
INORGANIC PHOSPHATE ESTIMATION (AFTER ALLEN (1)) After illumination reactions were stopped either by the addition of a small volume of 60 % perchloric acid or preferably by the addition of 9.5 ml of water containing perchloric acid, sodium metabisulphite and amidol. In either case the additions were such that the tubes contained the reaction mixture, 0.15 ml of 60 % perchloric acid and 0.15 ml of a solution containing sodium bisulphite (20 % w/v) and amidol (2,4-diaminophenol hydrochloride, 1 % w/v) in a final volume of 9.8 ml. Ammonium molybdate (0.15 ml of 8.3 % w/v $(NH_4)_6Mo_7O_{24}4H_2O$) was then added and the tubes centrifuged to remove the protein precipitated by the acid. After 5 minutes the intensity of the blue color which developed in the supernatant was determined by readings at 660 mμ. The quantity of phosphate was then obtained by reference to a standard curve.

The disappearance of inorganic phosphate has been taken to represent ATP formation from ADP. The inorganic phosphate content of reaction mixtures hydrolyzed for 7 minutes in *N* HCl before and after illumination was the same. The observed decrease in inorganic phosphate before hydrolysis therefore corresponds to an increase in "7 min" organic phosphate.

In the plants used chloroplast preparations showed no ATPase activity. In contrast, chloroplast preparations from leaves of barley seedlings have a high ATPase activity. The supernatant from a chloroplast preparation from spinach also gave smaller particles, when spun at 20,000 G, which showed a marked ATPase activity.







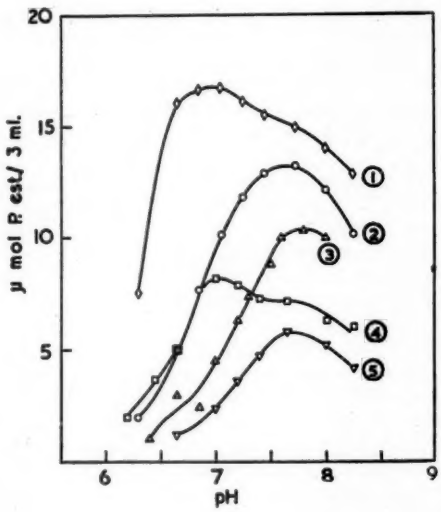


FIG. 1 (*top, left*). Water bath and illumination.

FIG. 2 (*top right*). Varying concentrations of added cofactors. The rate is expressed as a percentage of the maximum in each set (150 to 200 μM/phosphate/mg chlorophyll/hr). For reaction mixture see Materials and Methods.

FIG. 3 (*bottom left*). Progress curve of disappearance of inorganic phosphate. Reaction mixtures contained in a total volume of 0.3 ml, inorganic phosphate, 1.65 μM; ADP, 1.5 μM; FMN, 0.003 μM, ascorbate, 1 μM; chloroplast suspension, equivalent to 0.05 mg chlorophyll; TRIS-NaCl buffer at pH 7.2. A shows increase of phosphate in dark; B shows initial level of phosphate; and C difference between A and final level of inorganic phosphate.

FIG. 4 (*bottom right*). Activity of phosphorylation in relation to pH. For reaction mixtures see Materials and Methods. Curve 1: whole chloroplasts with pyocyanine, 10 min illumination; 2: whole chloroplasts with FMN + K_3 10 min illumination; 3: broken chloroplasts stored for 4 hours at 0° C with pyocyanine 6 min illumination; 4: broken chloroplasts with pyocyanine 4 min illumination; and 5: broken chloroplasts with FMN + K_3 4 min illumination.

Results

The activity of a cofactor, such as K_3 or FMN in relation to phosphorylation shows an optimum concentration in the region 10^{-5} M, and after some preliminary trials the experiments were carried out with a series of substances at this range. The results of experiments in air are shown in table I. The very wide range of oxidation-reduction potential in the characteristic potentials of the reagents at pH 7 is shown by data in the last column. Of all the substances tried phenazine methosulphate appeared to be the most active. (This was also found independently by Avron and Jagendorf (12)). We noticed however that the dilute stock solution (10^{-4} M) rather rapidly became purple or brownish when standing even in quite a dull light. This in no way affected the activity. A sample was allowed to stand in the light, the pyocyanine extracted in chloroform, purified and added in a concentration corresponding to the original, phenazine methosulphate. The activity of the pyocyanine was the same as that given by the phenazine methosulphate, either before or after exposure to light. The formation of pyocyanine in light from phenazine methosulphate requires O_2, so that in order to see if the phenazine methosulphate was active it was necessary to test it in absence of O_2. The removal of oxygen to a degree of completeness required when a minute amount of the phenazine methosulphate at a concentration of 10^{-5} M is involved proved impracticable in a time during which the activity of the chloroplast preparation could be maintained. We were thus led to run the phosphorylation reaction in the presence of catalase and ethanol relying on the Mehler reaction (14) to remove the last traces of O_2 by forming acetaldehyde. The presence of catalase and ethanol in vacuo did not affect the rate of phosphorylation with pyocyanine or with preilluminated phenazine methosulphate as compared with the rate in air.

Table I

List of "co-factors" used in phosphorylation

Activating agent	Potential	Rate
1,2-Naphthoquinone-4-sulphonate	+0.245	37
1,4-Naphthoquinone-3-sulphonate	+0.167	49
Ascorbate	+0.050	17
Juglone	+0.023	30
2-Methyl-1,4-naphthoquinone	+0.020	60
Methylene blue	+0.011	24
2,7-Dichlororesorufin	−0.006	61
Pyocyanine	−0.011	100
Resorcin blue	−0.060	28
Napthazarine	−0.105	53
FMN	−0.200	62
Dimethyl safranin sulphonate	−0.220	53
Anthraquinone-2-sulphonate	−0.250	52
Methyl viologen	−0.455	54

The potential is the characteristic potential in volts at pH 7. The rate is expressed as a percentage of the maximum (80 μM phosphate/mg chlorophyll/hr).

Table II

Comparison of Effects of Phenazine Methosulphate and Pyocyanine on Photosynthetic Phosphorylation

	μM P est/mg chl/hr	
	anaerobic	*aerobic*
Phenazine methosulphate	12	570
Pyocyanine	430	400*

Reactants: ADP and inorganic phosphate, 1.5 μM; $MgCl_2$, 10^{-3} M; TRIS, 0.05 M, pH 8.1; ethanol, finally 3 %; catalase, 0.03 × 10^{-3} M; ascorbate 10 μM; phenazine methosulphate or pyocyanine, 10^{-5} M; chloroplasts, broken, 0.0075 mg chlorophyll; in a final volume of 0.3 ml.

* A value of 1000 has been obtained at 15° using similar reaction mixtures.

If the experiment was set up in the dark and the O_2 removed as completely as possible then no increase in photosynthetic phosphorylation was obtained with phenazine methosulphate unless it had been previously exposed to light in the presence of air.

A further experiment has recently been carried out by Dr. F. L. Bendall with chloroplasts of *Chenopodium bonus-henricus* L. the result of which is shown in table II.

The data given in table III show a comparison between FMN and phenazine methosulphate. There is an indication of a short lag period in the case of the latter of about 1 minute which could represent the conversion to pyocyanine.

The effect of varying concentrations of added cofactors for phosphorylation is shown in figure 2. In the case of the magnesium ion the chloroplast preparation was not free from magnesium so that the effect of addition of Mg^{2+} is only shown at the higher concentrations. The broken curve is extrapolated.

In figure 3 a progress curve is shown and the slight increase of inorganic phosphate which may have been released from ADP in the corresponding dark experi-

Table III

Progress Curve—Comparison of Phenazine Methosulphate with FMN and K_3

Time (min)	A	B
2	2.0	2.8
4	5.4	3.8
6	7.8	4.8
9	12.0	4.4
12	13.8	7.4
15	18.4	10.2
19	18.8	11.4
24	24.4	11.2
30	24.4	12.6

Results as μM phosphate esterified/mg chlorophyll. Broken Swiss chard chloroplasts. A: 10^{-5} phenazine methosulphate. B: 10^{-5} FMN and 10^{-5} K_3.
Other reactants as indicated in Materials and Methods.

ment is shown by the sloping line lying above that indicating the theoretical total uptake for the added ADP. The disappearance of inorganic phosphate, which corresponds with the formation of ATP from ADP is nearly linear until all the added ADP is converted to ATP.

The effect of changes in pH on the amount of inorganic phosphate esterified in a short interval of time is shown in figure 4. With broken and unbroken chloroplasts there is 1 maximum with FMN and K_3 while with pyocyanine the curves suggest a superposition of 2 maxima, one corresponding with that in the former curves at pH 7.7 the other, showing a higher activity at pH 7. When the broken chloroplasts were kept at 0° C (in absence of ascorbate) for 4 hours the activity measured with FMN and K_3 was practically zero. The same sample measured in the presence of pyocyanine showed activity with a single maximum at pH 7.7. The curves suggest that there may be more than 1 site for the phosphorylation reaction as suggested by Wessels (16, 17) from his study of inhibitors with FMN and K_3. This, however, would not be the only possible interpretation of the present data.

Discussion

The results obtained by addition of various substances as co-factors for phosphorylation agree with the work (actually carried out simultaneously) of Jagendorf and Avron (12). Further we found that the addition of ascorbate was not always necessary though it did diminish the rate of loss of activity on storage of the chloroplast preparations at 0° C. Here again there is agreement in the results obtained in both Arnon's (5) and Jagendorf's (12) laboratories and with those of Wessels (16, 17).

The question as to whether phenazine methosulphate itself is active or whether the activity is due to its photochemical transformation to pyocyanine (which requires O_2) is still controversial. Our evidence, which was obtained using concentrations in the region of 10^{-5} M phenazine methosulphate, is in favor of the active agent being pyocyanine, rather than phenazine methosulphate. The concentration of pyocyanine for half activation is greater than that for FMN or K_3, but the maximum rate of phosphorylation for pyocyanine with some chloroplast samples is significantly higher. We were not able to observe any direct reduction of pyocyanine with chloroplast preparations, based on a bleaching of the color. There is, however, indirect evidence (Davenport, private communication) that pyocyanine can be rapidly reduced by illuminated chloroplast preparations; we are led to the conclusion that it can also be rapidly reoxidized by a photochemically induced precursor of free O_2 in the chloroplast system and hence, as will be made clear later, it would be supposed to have ideal properties for establishing the photophosphorylation—termed by Arnon as cyclic—which is dependent neither on the presence of O_2 nor on the production of O_2.

It now therefore becomes clear, as indeed Arnon has already emphasized (6) that there is no necessity for the direct utilization of molecular oxygen in the so called "cyclic phosphorylation." Originally (9) it was proposed that the reduced product formed in the light might be reoxidized by molecular oxygen to give a system similar to that operating in chemosynthetic organisms. Now, the important discovery by Arnon's group that O_2 production and reduction of H-acceptors is coupled with phosphorylation disposes of the need for a separate reoxidation system supplying the active phosphate groups. Also this discovery tends to show that, apart from Mg^{2+}, the co-factors used in the artificially constituted phosphorylating system need not be necessarily specific, just as in the case of the original H-acceptors for oxygen production. In other words, they are not in the strict sense "co-factors" at all. The phosphorylation reaction itself now appears as a part of the normal photochemically induced H-transfer. Of course it is essential to find out what are the substances in the cell responsible for establishing the photochemically driven transport, but until the nature of the coupling between reduction and phosphorylation has been analyzed the present argument remains unaffected. It does not follow from the argument that all substances capable of being reduced by illuminated chloroplast preparations would be capable of initiating phosphorylation. The coupling between reduction and phosphorylation can be abolished and an active agent can become inhibitory at higher concentrations so that the affinities relating to the chloroplast system have to be in a suitable range.

In the original experiments of Arnon, Whatley and Allen (2) with phosphorylation in the presence of ascorbate and magnesium ion only, O_2 was found to be necessary. Our experiments confirmed this, and also showed that the initial activity was maintained only for a short period. We did not determine whether the loss of activity was due to a distruction of an endogenous H-acceptor or inactivation of the whole system. In the more active systems when FMN and K_3 were added, the phosphorylation reaction was found by Arnon's group (3, 18) to progress better or slightly better in the absence of O_2. More recently, systems have been used (that with pyocyanine may serve as example) where no difference is observed between the rates observed in the presence and absence of O_2. On the basis of a requirement for establishing H-transport in the illuminated chloroplast system by catalytic amounts of added material, these differences could readily be explained as follows. The added material can be represented by X which is reduced to XH_2 in light and has to be continuously oxidized to X again so as to maintain the H-transport in the system. If XH_2 can only be oxidized in the presence of free O_2 then the system will require O_2 for activity. If XH_2 can be oxidized rapidly by the photochemical system in a stage prior to the liberation of O_2 then the system would be equally active in air or anaerobically. If XH_2 is also oxidized by O_2 and gives H_2O_2 this will result in a "Mehler reaction." The H_2O_2 is likely to cause loss of activity if -SH groups become oxidized. Thus in this case even in the pres-

ence of ascorbate, an improvement could result when O_2 is removed.

It may perhaps seem to some workers too sweeping a generalization to regard the phosphorylation reaction as forming a part of the photochemically induced H-transport system in the chloroplast. This would imply that differences in behavior of the so-called co-factors for phosphorylation are being governed by their reaction with the terminal H-donor in the chloroplast preparation. The problem is obviously simplified when phosphorylation can be studied in relation to a stoichiometric reduction of an H-acceptor. When a catalytic amount of an H-acceptor is used it introduces the further problem of reoxidation which can be supposed to occur by alternative mechanisms. The conclusion presented by Geller and Gregory (8) in relation to hydrogen transport in their work on phosphorylation with Rhodospirillum extracts may in part be applicable to the chloroplast system in the green plant.

Summary

Several substances have been found to increase phosphorylation at low concentration (10^{-5} M) in experiments carried out in air.

Pyocyanine and phenazine methosulphate were found to be the most active of the substances tested. Pyocyanine required a concentration of 6.3×10^{-6} M to give one half the maximum activity which was given with 1×10^{-4} M. FMN and K_3 were found to give full activity at 10^{-5} M and one half activity at 5×10^{-7} M. It was concluded that the activity observed with phenazine methosulphate was due to its rapid conversion to pyocyanine in light. The effect of changes in pH and of storage times of chloroplast preparations in the system containing pyocyanine showed marked differences from the system containing FMN and K_3.

The authors would like to thank Professor D. Keilin for a catalase preparation and for a sample of phenazine. They would also like to thank Dr. D. E. Green for a small quantity of phenazine methosulphate and Dr. S. Aronoff for the naphthoquinone sulphonates.

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PHOTOREDUCTION OF CHLOROPHYLL A IN THE PRESENCE OF ASCORBIC ACID IN PYRIDINE SOLUTIONS [1, 2]

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Over the last decade, Russian investigators—among whom Krasnovsky, Evstigneev, and Gavrilova are most prominent—have published many papers describing the photoreduction of chlorophyll in dilute solutions. Since the photoreduction has been reported to be reversible and energy-storing, the reaction is of great interest because it could be related to the primary photochemical process of photosynthesis. This paper describes experiments carried out after initial attempts by the writer failed to reproduce certain of the Russian findings.

Krasnovsky and Brin (5) reported that, when an oxygen-free pyridine solution of chlorophyll a (10^{-5} M) and ascorbic acid was illuminated with red light, the blue-green color characteristic of chlorophyll faded away and a pink solution was formed: the absorption bands of chlorophyll disappeared and a new absorption band with a maximum at 523 mμ appeared. Lynch and French have confirmed this observation. Krasnovsky et al (3, 4, 5) found that, with a moderate concentration of ascorbic acid (0.05 M) and in the absence of water, the bleaching was reversible—i.e., in the dark following bleaching, chlorophyll was regenerated and a blue-green color was restored. In solutions containing water or more ascorbate (0.10 M), the bleaching was irreversible since pheophytin a rather than chlorophyll was generated in the dark. The Russian workers apparently regarded the formation of pheophytin as an unimportant secondary process unrelated to the photoreduction.

The results of preliminary experiments of the writer differed significantly from those of the Russians'. In dry pyridine solutions, no bleaching was detected at all. When a certain amount of water was added to the samples, a rapid bleaching resulted but colorless rather than pink solutions were formed; moreover, no reversibility could be demonstrated because the bleached chlorophyll was quantitatively converted into pheophytin in the dark following illumination. These findings thus failed to corroborate the existence of a pink photoreduced chlorophyll and failed also to show any reversible reaction at all. Since the Russian studies were not only convincing in their extent, but also important in providing evidence for the only known, reversible, light-sensitized, energy-storing redox reaction of chlorophyll, a thorough reinvestigation was undertaken. Part of the results of this study are reported in this paper.

MATERIALS AND METHODS

Chlorophyll a was prepared by Jacob and Holt's method (2). Merck ascorbic acid and Fisher reagent grade pyridine were used. Safranine O (same as safranine T, CI 841) was an 82% preparation of Hartman-Leddon Co., Philadelphia; the impurity was presumed to be salt.

Solutions of chlorophyll and of ascorbic acid in pyridine were always freshly prepared. (Solutions of ascorbic acid are quite unstable as shown by the development of a pink color after a few hours standing.) To remove oxygen, solutions were sometimes swept with Matheson "prepurified" nitrogen (which contains less than 8 ppm of oxygen). More often, the samples were de-oxygenated by evacuation : after adding excess pyridine, the sample was evacuated and dissolved gases and pyridine vapor were pumped off from the boiling solution until the required volume of solution was reached.

The sample vessel consisted of a pyrex cuvette ($1 \times 1 \times 4$ cm), clear on all 4 sides, to which was attached an upper portion containing an enlarged cavity to facilitate boiling during evacuation, a side arm, and a ground glass stopper with a tube for attaching to a vacuum pump.

Actinic light was produced by a Koehler projection system consisting of the following elements. The source was a 1000-watt projection lamp operated from a Variac transformer, which was, in turn, powered by a 2000-watt Sola voltage regulator. A condenser of 6-inch diameter formed an enlarged image of the lamp filaments on a projection lens. At the condenser was an adjustable diaphragm, an image of which was focused on the sample vessel. Between the condenser and projection lens, was an infra-red absorbing filter consisting of a piece of Solex glass, a piece of window glass, and a 1.5 cm thick layer of water. Between the projection lens and the sample, colored glass or interference filters could be mounted. A Schott BG 21/7 infra-red absorbing filter was always used in conjunction with colored glass filters, but not with interference filters. In some experiments, the sample vessel was kept in air, in others in a water bath; in either case, no heating of the sample was detected. With the vessel in air, a thermopile could be positioned at the back of the vessel allowing direct measurement of the transmitted intensity. With the vessel in the water bath, the thermopile was positioned about 2.5 inches behind

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[3] This work includes portions of the writer's thesis which was presented to the graduate college, University of Illinois, in August 1958.

the sample; in this case, the actual intensity at the vessel was calculated as the product of the observed intensity behind the vessel and an experimentally determined proportionality factor.

The thermopile was an Eppley, bismuth-silver, non-evacuable model, which, with a Multiflex galvanometer (60-ohms internal resistance, 0.02 microamps/mm sensitivity), was calibrated with a secondary standard lamp according to the procedure recommended by the National Bureau of Standards. The calibration showed that a deflection of 1 mm corresponded to an incident intensity on the thermopile of 26.2 microwatts/cm².

In some experiments, the rate of photochemical reaction in an irradiated sample was determined by following, with the thermopile, the transmission of actinic light, which had been rendered roughly monochromatic by an interference filter having maximum transmission at the wave length of the absorption maximum of chlorophyll (670 mμ). In other experiments, the course of the reaction was determined by following the transmission of a low intensity beam of light passing through the sample perpendicularly to the actinic beam; in this "cross-beam" technique, the source was a battery-operated, 6-volt, 18-amp, ribbon filament lamp, the filament of which was imaged by lenses first on the sample and then again on the entrance slit of a Bausch and Lomb monochromator. (The monochromator had a focal length of 250 mm and a dispersion of 6.6 mμ per mm of slit width; in general, slit widths of 0.30 mm or less were used). The intensity of monochromatic light leaving the exit slit was measured with a battery-powered, 931-A, RCA multiplier phototube, the output of which was read with a galvanometer.

Results

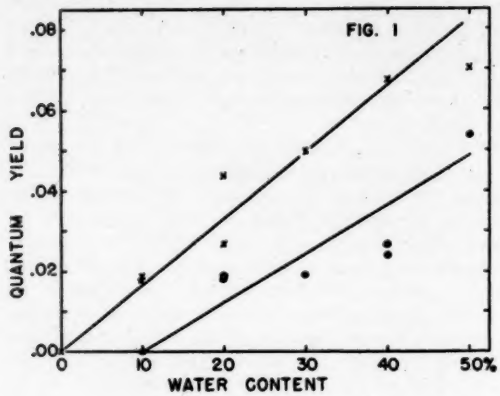
Water Requirement: Contrary to the Russian findings, no bleaching of chlorophyll, in the presence of asorbic acid, was observed in dry pyridine solutions. When water was added to the samples, irradiation produced rapid bleaching, but the resulting solutions were colorless or faintly green rather than pink. (In a letter to the writer, Linschitz has also reported that water is necessary for bleaching.) In the dark following irradiation, pheophytin rather than chlorophyll was generated in the bleached solutions. Since pheophytin has a maximum extinction coefficient of only about one half that of chlorophyll, only part of the original absorption at 670 mμ was restored in the dark. The absence of bleaching in dry pyridine not only contradicted the Russian reports, but also cast doubt on the alleged reversibility of the reaction, since the regeneration of chlorophyll in the dark was reported to be demonstrable only in dry pyridine.

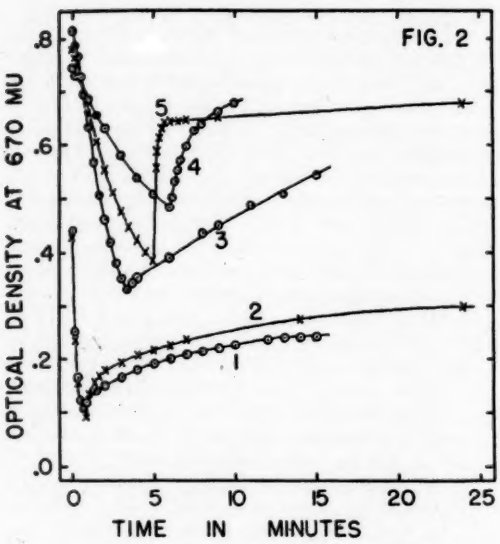
The author employed ordinary, reagent grade pyridine while the Russians used pyridine purified by fractional distillation of a pyridine-benzene mixture (3). An explanation of the requirement of water for the bleaching of the writer's samples could be that water inactivates unknown bleaching inhibitors, present in trace amounts in ordinary pyridine. To test this hypothesis, the quantum yields of bleaching as a function of water content were compared for samples prepared from ordinary pyridine and from purified pyridine.

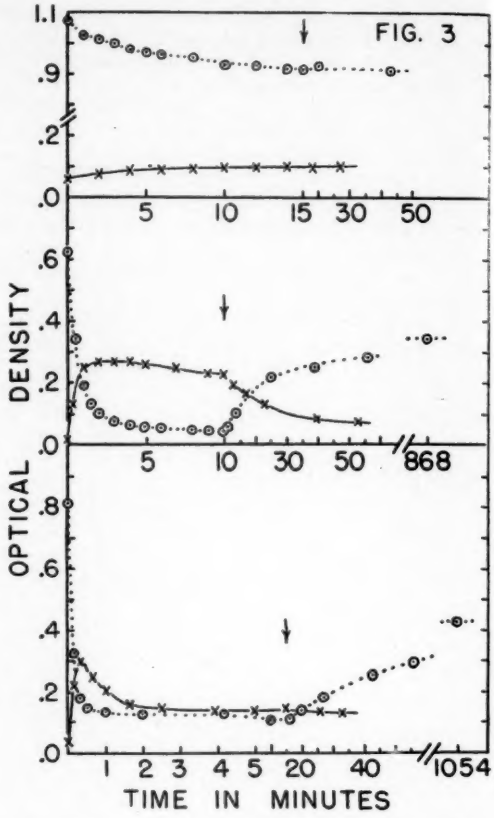
"Ordinary" pyridine was Fisher's reagent grade. "Purified" pyridine was prepared by fractional distillation. The still consisted of a wire-wound reflux column to which a distillation head and condenser were attached. The still was charged with 2 pints of Fisher's reagent pyridine. One half of the charge was distilled off and discarded; one half of the charge remaining was collected as the purified fraction, and the remaining one quarter of the initial charge was discarded. Coulson et al (1) showed that this procedure yields pyridine of low water content.

The samples contained chlorophyll a (6 to 8×10^{-6} M), ascorbic acid (0.10 M), water, and pyridine. O_2 was removed by flushing with N_2 for about 15 minutes before beginning illumination. The apparatus consisted of the Koehler system without the "cross-beam". A Farrand interference filter with maximum transmission at 672 mμ was used to isolate the red, actinic light. The thermopile-galvanometer pair was used to determine both the incident intensity (in quanta/(cm² × sec)) and the sample transmission as a function of time after beginning the irradiation. Since the interference filter passed too broad a band of red light to permit converting measured transmission values into optical density values at 672 mμ, an empirical calibration curve was prepared, using solutions of different concentrations of chlorophyll, by plotting the transmission of actinic light against the optical density at 670 mμ determined with a Beckman DU spectophotometer. With this calibration curve, the sample transmission was converted into the optical density at 670 mμ, and, using the coefficient of 85×10^3, the concentration of chlorophyll was computed. After plotting the latter as a function of irradiation time, the initial rate of change of concentration was estimated. Because the bleaching was often too rapid to determine directly the initial transmission of the sample, this quantity, required in order to calculate the initial absorbed light intensity, was also determined from the calibration curve, using the optical density at 670 mμ measured in the Beckman before irradiation.

The quantum yields thus computed are subject to several errors. The molar extinction coefficient of chlorophyll a in pyridine is, according to estimates made after these results were obtained, nearer to 79×10^3; slightly lower values still may apply to aqueous pyridine solutions. The products of chlorophyll bleaching were assumed to absorb no actinic light; both the time course of transmission as well as other evidence show that the products of bleaching may have significant absorption. Finally, evidence to be presented later indicates that the nature of the products of bleaching depends on the water content. On account of these factors, the above calculated yields may be too low—perhaps by as much as a factor of 2.







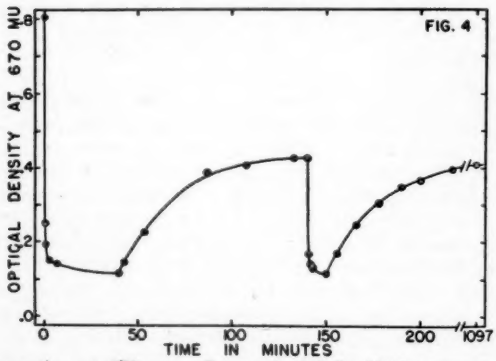


FIG. 1. The quantum yield of bleaching as a function of the water content of the samples. Crosses—samples prepared with pyridine purified by fractional distillation. Circles—samples prepared with Fischer's reagent-grade pyridine. Samples contained 8×10^{-6} M chlorophyll a, 0.10 M ascorbic acid, water, and pyridine; oxygen was removed with a stream of nitrogen.

FIG. 2. The bleaching and recovery in the dark of the absorption at 670 mμ. Curves 1 and 2; 30 % aqueous pyridine: 1—no oxidant added; 2—safranine (10^{-3} M) added at beginning of dark period. Curves 3, 4 and 5; 10 % aqueous pyridine : 3—no oxidant added; 4 and 5—safranine added at beginning of dark period, resulting concentrations 1.0×10^{-5} and 1.0×10^{-3} M respectively.

FIG. 3. Optical density at 670 mμ (circles) and 520 mμ (crosses) during bleaching and recovery in the dark in 0 % (top), 10 % (middle), and 30 % (bottom) aqueous pyridine. Samples contained 8 to 14 $\times$ 10^{-6} M chlorophyll a, 0.010 M ascorbic acid, water, and pyridine and were evacuated to remove oxygen. Arrows show when actinic light was turned off.

FIG. 4. The reversible bleaching of pheophytin a. Sample initially contained chlorophyll a (10^{-5} M) and ascorbic acid (0.10 M) in 30 % aqueous pyridine; oxygen was removed by evacuation. Initial actinic irradiation rapidly destroyed the red absorption band of chlorophyll and gave rise to a photo-derivative having about 0.15 of the extinction of chlorophyll. In the dark pheophytin was formed. When the solution was bleached a second time, the extinction fell again. In the dark, pheophytin was regenerated.

The data (fig 1.) confirm the preliminary finding that water is required for chlorophyll bleaching. They show also that the dependence of the quantum yields of initial bleaching upon water content is similar in the series of solutions prepared with ordinary pyridine and with purified pyridine. (The somewhat smaller concentration at which significant bleaching occurred with purified pyridine probably indicates that a little water vapor dissolved in the distillate during the purification. The difference in slope may be due to a difference in temperature, the 2 series of data having been obtained on different days). The similar dependence of the yield upon the water content in both series indicates that water does not cause rapid bleaching by overcoming the effects of inhibitors present in the solvent. More probably, water enters directly into the mechanism of chlorophyll bleaching. The writer cannot explain Krasnovsky's explicit statements that the bleaching of chlorophyll occurs in the absence of water, except by supposing that his "dry" pyridine contained water to the extent of 5 % or more by volume.

SAFRANINE-ACCELERATED REGENERATION OF RED ABSORPTION IN BLEACHED SOLUTIONS: An attempt was made to confirm the Russian reports that the addition of safranine to bleached chlorophyll solutions (which were pink according to the Russians, but colorless or faintly green in our case) accelerated the regeneration of a red absorption band. The apparatus consisted of the Koehler projection system for actinic light and the "cross-beam" system for monitoring the optical density of the sample at 670 mμ.

The samples contained chlorophyll a (7×10^{-6} M), ascorbic acid (0.10 M), water, and pyridine; oxygen was removed by flushing with nitrogen. After bleaching, a small volume of a solution of safranine T (in the same aqueous pyridine solvent as the chlorophyll solution) was added, and the regeneration of the absorption at 670 mμ was followed.

The changes in optical density at 670 mμ during bleaching and restoration in the dark are shown in figure 2 for 10 and 30 % aqueous pyridine preparations, with and without safranine addition following bleaching. In 10 % aqueous pyridine, in agreement with Krasnovsky's findings, safranine T in concentrations of 10^{-3} M and 10^{-5} M markedly accelerated the regeneration of red absorption. In 30 % aqueous pyridine, in contrast, safranine did not accelerate the regeneration of the absorption in the red, except, perhaps slightly, for a very short period immediately following its addition.

These findings can be explained by postulating that, in aqueous pyridine, reduced chlorophyll arises in light, but is removed by a dark reaction. In 10 % aqueous pyridine, the removal of the reduced chlorophyll would be relatively slow, so that an accelerated regeneration of a red absorption band could be observed when an oxidant is added immediately following bleaching. In 30 % aqueous pyridine, the conversion of reduced chlorophyll into a substance, not oxidized by safranine, would occur so rapidly that by the end of the illumination period no reduced chlorophyll would remain, and, therefore, no accelerated regeneration of a red absorption band could be seen. The absence of a visibly pink color in bleached solutions must mean that sizeable accumulations of reduced chlorophyll do not arise. In 30 % aqueous solutions, the rapid removal of reduced chlorophyll must be the primary explanation; in 10 % aqueous pyridine, in which some reduced chlorophyll does accumulate (as shown by the safranine-caused oxidation), the rate of bleaching is low and much unbleached chlorophyll remained, thus maintaining a green color.

FORMATION OF PHEOPHYTIN: Preliminary experiments showed that, in 30 % aqueous pyridine, the regeneration of bleached solutions in the dark yielded chlorophyll and pheophytin in concentrations, the sum of which equalled the concentration of chlorophyll before bleaching. Moreover, it was found that all of the bleached chlorophyll was converted into pheophytin in the dark; any residual chlorophyll found after irradiation and a subsequent dark period, was apparently not bleached at all. The question arose, therefore, as to what part of the observed bleaching, which the Russian workers attributed to the formation of reduced chlorophyll, was actually associated with the formation of pheophytin.

It is well-known that strong acids convert chlorophyll into pheophytin in the dark. Moreover, Rabinowitch has shown that, with weak acids, pheophytinization is accelerated by light (8). One way of forming pheophytin in irradiated solutions of chlorophyll and ascorbic acid is, then, by a light-accelerated reaction, in which hydrogen ions, formed by the dissociation of ascorbic acid, replace magnesium in chlorophyll. On the other hand, pheophytin might also arise in a reaction between photoreduced chlorophyll and hydrogen ions, followed by an oxidation. To determine which of these two possible reaction paths accounted for the formation of pheophytin in illuminated solutions of chlorophyll and ascorbic acid, photopheophytinization was studied in the presence of reducing and non-reducing acids.

Oxygen-free solutions of chlorophyll (7×10^{-5} M) and acid (0.10 M) in 30 % aqueous pyridine were prepared. The samples were irradiated with red light (the initial rate of excitation of chlorophyll was 5.0×10^{-6} moles/(liter × sec)), and the rate of decrease of the extinction at 670 mμ was followed. Before and after the actinic illumination, the absorption spectrum of each sample was measured.

With succinic, malic, and malonic acids, irradiation converted chlorophyll into pheophytin directly; there was no further reaction in the dark after illumination. With ascorbic acid, as has already been stated, chlorophyll was converted by irradiation into a nearly colorless intermediate, from which pheophytin was generated in the dark. Such behavior indicates that the pheophytinization of chlorophyll in the presence of ascorbic acid differs from the photoactivated pheophytinization with non-reducing weak acids. This view was further supported by the finding that

the initial rate of bleaching of chlorophyll was 10 to 30 times faster with ascorbic acid than with the nonreducing acids succinic, malic, and malonic, even though the latter are up to 50 times stronger acids than the former. It is clear therefore, that the rapid formation of pheophytin in our experiments is associated with the reducing properties of ascorbic acid. The explanation must be that pheophytin arises mainly from the reduced chlorophyll derivative formed in light, not from chlorophyll itself.

Whereas solutions of either chlorophyll or pheophytin in aqueous pyridine possess strong absorption bands at about 670 mμ, the bleached solutions absorbed but weakly at this wave length, both in 10 % aqueous pyridine (where the safranine-caused acceleration of the regeneration of red absorption may be taken to indicate the presence of reduced chlorophyll) and in 30 % aqueous pyridine (where safranine had little effect on the rate of recovery of a red absorption band). It is necessary, therefore, to postulate the existence, in addition to that of reduced chlorophyll, of a 2nd bleached derivative with weak absorption at 670 mμ. This substance is apparently formed when pink reduced chlorophyll disappears from solution. Since pheophytin was generated in the dark, the simplest hypothesis is that the substance in question is reduced pheophytin, formed by a reaction between reduced chlorophyll and hydrogen ions.

Changes in Optical Density at 520 and 670 mμ During Bleaching and a Subsequent Dark Period: The hypothesis that pink reduced chlorophyll, reported by the Russians to have an absorption maximum at 523 mμ, appears during bleaching but is rapidly converted into nearly colorless reduced pheophytin was supported by a study of the changes in optical density at 520 and 670 mμ. Samples of chlorophyll (8 to 14×10^{-6} M) and ascorbic acid (0.010 M) were prepared in 0, 10, and 30 % aqueous pyridine and thoroughly evacuated to remove oxygen. The samples were bleached with red light of an intensity of 6×10^{16} quanta/(cm^2 × sec); the initial rate of excitation of chlorophyll exceeded 15 μM/(liter × sec). The optical density was measured with the "cross-beam". Since the optical density at 520 and 670 mμ could not be measured simultaneously, different samples of identical composition were used.

In the light, the time courses of the optical density at 670 mμ (fig. 3) show a very slow bleaching in dry pyridine, a rapid bleaching in 10 % aqueous pyridine, and an even more rapid bleaching in the 30 % aqueous sample. In the 10 % aqueous preparations, the optical density at 520 mμ increased to a maximum in about 2.5 minutes; then gradually declined, even though the irradiation continued. In the 30 % aqueous system, the optical density at 520 mμ increased to a maximum in about 20 seconds, then rapidly dropped to an essentially constant level.

In the dark, after illumination, no change occurred in the optical density at either 520 or 670 mμ in dry pyridine, suggesting that, rather than photoreduction, a slow irreversible photo-degradation of chlorophyll may have occurred. In 10 and 30 % aqueous pyridine, the optical density at 670 mμ increased in the dark, finally reaching about 0.55 of that of the original chlorophyll solution. The restoration of this fraction of the original chlorophyll absorption at 670 mμ indicates that practically all of the chlorophyll initially present was converted into pheophytin. (The ratio of the extinction coefficients of pheophytin and chlorophyll at 670 mμ is about 0.55.)

The transient appearance of an increased absorption at 520 mμ during bleaching supports the hypothesis that the 1st-formed intermediate is the pink reduced chlorophyll. In 10 % aqueous pyridine, the maximum absorption at 520 mμ, occurring after 2.5 minutes irradiation, was about 0.4 of that of chlorophyll at 670 mμ before illumination; since the subsequent decrease in the absorption at 520 mμ was relatively slow, the extinction coefficient of the pink derivative at 520 mμ is probably about 0.4 that of chlorophyll at 670 mμ. Since the optical density at 670 mμ fell to a low value during bleaching, the pink derivative can have but little absorption at this wave length.

In 30 % aqueous pyridine, the short-lived spike in the time course of the optical density at 520 mμ indicates that the pink reduced chlorophyll quickly disappeared. The persistence of significant absorption at both 520 and 670 mμ indicates that the 2nd bleached product has extinction coefficients at both wave lengths of about 0.15 of that of chlorophyll at 670 mμ.

The time courses thus verify the hypothesis that 2 intermediates form when chlorophyll is bleached. The 1st has extinction coefficients of about 32×10^3 at 520 mμ and 8×10^3 at 670 mμ and is probably the pink derivative described by the Russian authors. This 1st derivative disappears slowly in 10 %, quickly in 30 % aqueous pyridine, generating a product having extinction coefficients of about 13×10^3 at both 520 and 670 mμ. In the dark, pheophytin is formed from this 2nd product.

That the 2nd product is a derivative of pheophytin rather than of chlorophyll is shown by the following. In figure 4 is plotted the time course of the optical density at 670 mμ for an evacuated and illuminated solution of chlorophyll a (10^{-5} M) and ascorbic acid (0.10 M). The actinic light intensity was about 2×10^{16} quanta/(cm^2 × sec). The graph shows the disappearance of chlorophyll in the 1st minute of irradiation, followed by a period of very slowly decreasing optical density. During the latter period, the remaining small amounts of chlorophyll and its reduced derivative were converted into the 2nd intermediate. When the actinic irradiation ended, pheophytin was generated. During a 2nd cycle of bleaching and dark recovery, the pheophytin was bleached, yielding an intermediate again characterized by an optical density at 670 mμ equal to about 0.15 of that of the original solution of chlorophyll; in the dark, pheophytin was restored just as before. This behavior indicates that the 2nd intermediate is directly formed by the photoreduction of pheophytin, as well as indirectly by the

conversion of the pink reduced chlorophyll derivative. The reproducibility manifest in the 2nd cycle of bleaching and restoration in the dark indicates, additionally, that in aqueous pyridine, the bleaching of pheophytin is a reversible phenomenon.

SPECTRUM OF REDUCED PHEOPHYTIN: Since the photoreduction of pheophytin is completely reversible in the dark, the absorption spectrum of reduced pheophytin could be determined in a solution of pheophytin and ascorbic acid in 30 % aqueous pyridine maintained in a bleached, photostationary state by continuous illumination.

A sample containing chlorophyll a (10^{-5} M) and ascorbic acid (0.10 M) in 30 % aqueous pyridine and thoroughly evacuated to remove oxygen, was bleached with red light [intensity: 2×10^{16} quanta/cm^2 $\times$ sec)]. After the chlorophyll had been photoreduced and converted to reduced pheophytin, the absorption spectrum of the sample, maintained in the photostationary bleached state, was determined with the "cross-beam" apparatus. Measurements were begun approximately 10 minutes after the beginning of the actinic irradiation and required about 30 minutes to complete; during this time, the sample was irradiated continuously, except for periods of a few seconds each when the blank was substituted for the sample to check the 100 % transmission mark.

The absorption spectrum, thus determined (fig 5), shows no indication of the presence of chlorophyll a or pheophytin a, which have strong absorption maxima at 670 and 440 or 670 and 410 mμ, in aqueous pyridine. Nor is there a strong absorption band with maximum at 520 mμ to indicate the presence of reduced chlorophyll. Instead, the spectrum exhibits low maxima at 540 and 620 to 640 mμ and a high absorption maximum at about 370 mμ. A gradually decreasing absorption extends into the infra-red past 760 mμ. The ratio of the extinction coefficient of the bleached pheophytin a derivative at 670 mμ to that of chlorophyll a at the same wave length is about 0.15, in agreement with the estimates made previously from the time courses.

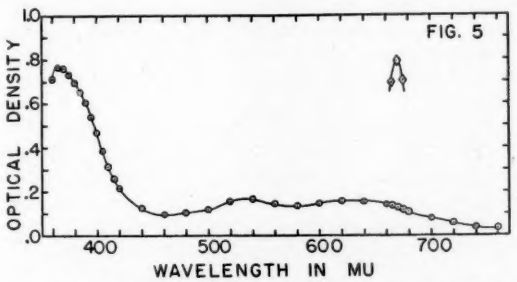


FIG. 5. Absorption spectrum of photoreduced pheophytin a. Before bleaching, the evacuated sample contained 10^{-5} M chlorophyll a, 0.10 M ascorbic acid, and 30 % aqueous pyridine. Circles—absorption spectrum of photoreduced pheophytin. Diamonds—absorption peak of the chlorophyll solution before bleaching.

The bleached pheophytin derivative is nearly colorless in 10^{-5} M solution; a blue-grey hue becomes quite obvious in more concentrated solutions.

The same spectrum (fig 5) is found when a solution of pheophytin a and ascorbic acid is bleached, proving conclusively that this spectrum is that of photoreduced pheophytin.

DEMONSTRATION OF THE PINK DERIVATIVE: Figure 3 shows that the first bleached product in the photoreduction of chlorophyll absorbs moderately strongly at 520, but only weakly at 670 mμ. This suggests that the derivative is the pink substance described by Krasnovsky.

In order to identify the first derivative more certainly with the pink substance, the absorption spectrum of the former was determined qualitatively by a rapid scanning technique.

For this work, the cross-beam apparatus was modified so that the sample transmission from 400 to 700 mμ could be scanned in about 15 seconds. The output of the photomultiplier, located at the exit slit of the monochromator, was placed on the y-axis of a type 304-A Dumont oscilloscope. On the x-axis was put the signal from a precision linear potentiometer, the setting of which was coupled through gears to the rotation of the wave length shaft of the monochromator. Thus, when the wave length shaft was rotated, the photomultiplier response to the various wave length components of the monitoring beam was traced on the oscilloscope screen as a function of the wave length.

In practice, 3 or more traces were recorded on each negative: a zero transmission (monitoring beam shut off); a 100 % transmission (a blank containing pure solvent in the beam); and 1 or more sample transmissions. True transmission spectra were not obtained, for no device was employed to correct for the wave length dependences of the monitoring beam intensity, the sensitivity of the photomultiplier, and the monochromator transmission. Two factors—insufficient lamp stabilization and a large time constant of the photomultiplier signal (about 0.01 second)—caused distortions and fluctuations in the traces such as precluded the calculation of true absorption spectra from the photographs. Despite these limitations, the transmission spectra sufficed to indicate qualitatively the extent of sample bleaching, the absorption characteristics of the 1st intermediate, and the nature of the products of the dark reactions.

Figure 6 shows the results of the irradiation of solutions of chlorophyll a and ascorbic acid in 0, 10, and 30 % aqueous pyridine. The samples were evacuated to remove oxygen and were irradiated for 1 to 3 minutes with red light of an intensity of about 0.1 microeinstein/(cm^2 $\times$ sec). In dry pyridine, the traces showing transmission before and after 3 minutes of actinic illumination nearly coincide; only in the region 500 to 540 mμ did a very small increase in the absorption of the irradiated sample produce significant departure from the "before irradiation" trace. The absence of significant bleaching in dry pyridine agrees

with the findings described previously. In 30 % aqueous pyridine, 1 minute of actinic irradiation destroyed large portions of the original absorption of chlorophyll at 440 and 670 mμ. In the middle range of the spectrum, a moderate increase in the absorption was registered, but no pronounced maxima or minima were evident. The absence of any striking features in the new absorption in the middle range is consistent with the presence of the reduced pheophytin derivative, which arises quickly in 30 % aqueous pyridine and has a rather featureless absorption spectrum. In 10 % aqueous pyridine, 1 minute of irradiation produced considerable bleaching of chlorophyll at 440 and 670 mμ, and formed a derivative having a strong absorption maximum at about 520 mμ. After 33 minutes in the dark, the 520 mμ absorption maximum had largely disappeared, and the increased absorption at 670 mμ and at about 410 mμ indicates the formation of pheophytin.

The absorption spectrum of the intermediate in 10 % aqueous pyridine, having a strong maximum at 520 mμ and little absorption in the red, is that of a substance pure solutions of which would be pink. Since the wave length of the absorption maximum of

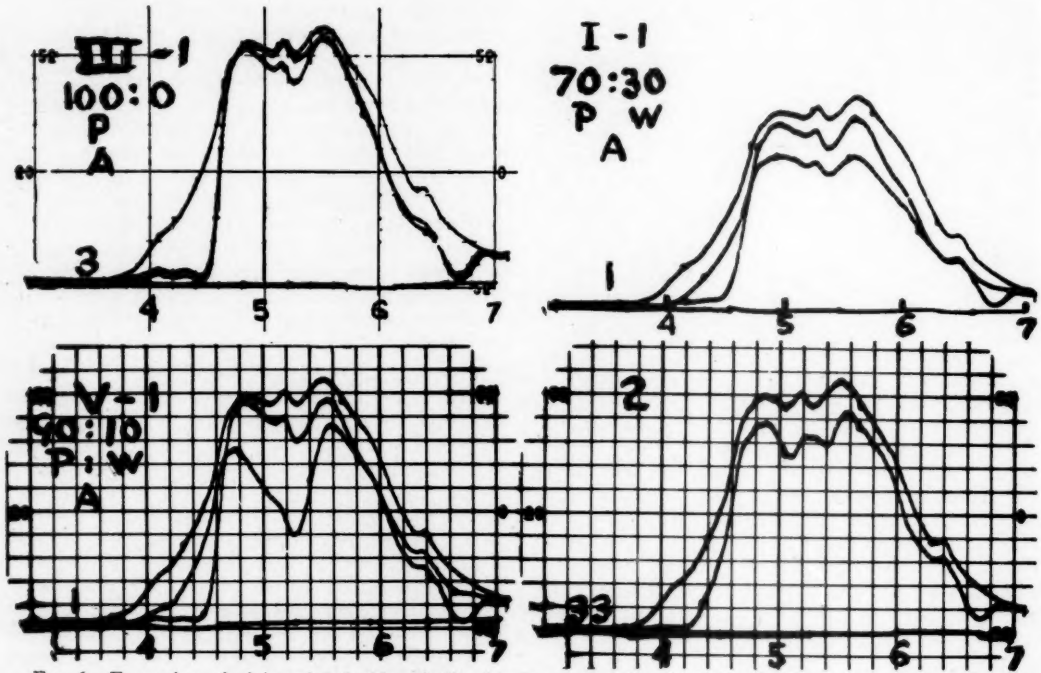


FIG. 6. Formation of pink reduced chlorophyll. Abscissa—wave length in hundreds of mμ. Ordinate—transmitted intensity (uncorrected for the wave length dependences of source output, monochromator transmission, and photomultiplier sensitivity.) Transmission between 300 and 700 mμ scanned in 15 seconds. In each of the 4 plates, the uppermost trace corresponds to "100 % transmission," the flat trace at the bottom corresponds to "0 % transmission" and the intermediate traces are "sample transmissions."

Upper left. Sample: chlorophyll and 0.10 M ascorbic acid in dry pyridine. The 2 sample transmission curves are "before actinic irradiation" and "after 3 minutes irradiation." There is very little difference between the 2 curves, showing that very little bleaching occurred.

Upper right. Sample: chlorophyll and 0.10 M ascorbic acid in 30 % aqueous pyridine. Traces for "before" and "after" 1 minute of irradiation. The original chlorophyll absorption at 440 and 670 mμ was largely destroyed by the irradiation; the bleached solution had increased absorption in the region 460 to 610 mμ, but no marked absorption maxima or minima are evident.

Lower left. Sample: chlorophyll and 0.10 M ascorbic acid in 10 % aqueous pyridine. Traces: "before" and "after" 1 minute of irradiation. The irradiation largely destroyed the chlorophyll absorption band at 440 and 670 mμ and produced a new pronounced absorption band at about 520 mμ.

Lower right. Same sample as *lower left*, after 33 minutes in the dark following irradiation. All of the 520 mμ absorbing substance has disappeared. Absorption in the violet and red has increased, but instead of the 440 mμ absorption of chlorophyll a, a 410 mμ maximum has appeared, indicating that pheophytin has been generated.

the intermediate is about 520 mμ and since the intermediate appeared in 10 % aqueous pyridine (the solvent in which a safranine-reducing derivative has been shown to accumulate), the intermediate must be the pink reduced chlorophyll derivative, described by Krasnovsky.

PINK SOLUTIONS: In order to form pink solutions, that is, to slow down the conversion of pink reduced chlorophyll into blue-gray reduced pheophytin, the concentration of hydrogen ions, formed by the dissociation of ascorbic acid, was diminished either by adding NaOH or by employing sodium or calcium ascorbate as the reductant. Observations were made on the speed of bleaching and the time to develop pink or blue-grey color in solutions initially containing chlorophyll (2×10^{-5} M), reductant, water, and pyridine. The solutions were de-oxygenated by flushing with nitrogen, and bleached with the unfiltered light from a 100-watt, frosted, tungsten lamp, placed at a distance of 2 inches from the sample.

In samples containing chlorophyll and ascorbic acid (0.10 M) in 4 % aqueous pyridine, NaOH in concentrations between 4.4×10^{-4} M and 0.04 M permitted, in light, the formation of pink solutions which lasted for 10 minutes or more. With concentrations of base smaller than 4.4×10^{-4} M, the blue-gray color of reduced pheophytin appeared after 1 minute of irradiation. In 15 % aqueous solutions, pink color appeared within 10 seconds with 0.0044 M NaOH, but changed to blue-gray after 2 minutes; in the absence of NaOH, no pink color arose; instead the blue-gray color appeared directly after about 10 seconds of illumination. In 30 % aqueous pyridine, blue-gray solutions appeared directly after 5 to 10 seconds of light; no pink colored intermediate stage was seen even with 0.044 M NaOH. NaOH in concentrations greater than 0.04 M elicited a separation of the solutions into two phases—one a pyridine solution of chlorophyll, the other a viscous, water-NaOH phase, in which ascorbic acid accumulated.

These results show that the addition of NaOH can bring about the formation of pink solutions in 4 and 15 % but not in 30 % aqueous pyridine. The explanation of this difference in behavior is probably that in 4 and 15 % aqueous pyridine, only a small portion of the ascorbic acid is ionized and the hydrogen ion concentration can, therefore, be substantially reduced by 0.004 M NaOH. In 30 % aqueous pyridine, most of the ascorbic acid is ionized and NaOH is insufficiently soluble to completely neutralize all of the hydrogen ion formed.

In 27 % aqueous pyridine, no bleaching of chlorophyll occurred in samples in which calcium or sodium ascorbate (0.010 M) was employed as reductant. If, however, a small quantity of HCl was also added (0.001 M), a rapid bleaching resulted, giving rise to pink solutions. The pink color persisted for about 1.5 minutes, then was replaced by blue-gray.

The absence of bleaching in the presence of non-acidified sodium or calcium ascorbate, suggests that the bleaching reaction requires the presence of some hydrogen ions. Too much acidity, however, prevents the formation of pink solutions by favoring the rapid formation of reduced pheophytin. Thus, pink solutions form only when an intermediate hydrogen ion concentration is provided, neither so low as to inhibit bleaching nor so high as to rapidly convert pink reduced chlorophyll into blue-gray reduced pheophytin.

DISCUSSION

The experiments reported here confirm, in pyridine in the presence of ascorbic acid, a photoreduction of chlorophyll, the primary product of which is pink reduced chlorophyll. The writer's findings differ, however, from those of the Russian's in the water requirement and the formation of pheophytin.

One explanation of the water requirement would be that the effective electron donor is the ascorbate anion and that the latter is formed (by the dissociation of ascorbic acid) in amounts increasing with the water content. Several pieces of evidence support this view. First, with phenylhydrazine as electron donor in toluene or ether solutions, no water is required for the photoreduction of chlorophyll; thus, the water requirement appears to be specific to the reductant ascorbic acid. (The photoreduction of chlorophyll in toluene and ether will be described in another paper.) Secondly, it is well-known that the singly-charged ascorbate anion is a stronger oxidant than the undissociated acid: in the presence of oxygen, acidic solutions of ascorbic acid are relatively stabile, while alkaline solutions are rapidly oxidized (7). Finally, ethanol can be substituted for water in support of the photoreduction with ascorbic acid in pyridine; thus, it appears to be a strong dieletric, not water specifically, which is needed for photoreduction.

The experiments described show that the formation of pheophytin is not the result of side reactions occurring independently of the photoreduction of chlorophyll but rather is the direct consequence of having pink reduced chlorophyll and hydrogen ions together. In fact, so quickly and easily does the pink substance undergo pheophytinization that the reaction can be recommended for the preparation of pheophytin. It is of interest that the magnesium ion of chlorophyll is made more labile by the photoreduction.

The following steps summarize the reactions indicated in this study.

1) $AH_2 + H_2O \rightleftharpoons AH^- + H_3O^+$

2) $Chl + AH^- \xrightarrow{h\nu} Chl_{red.}$

3) $Chl_{red.}$ + oxidant (such as safranine) $\longrightarrow$ Chl + reduced oxidant

4) $Chl_{red.} + 2H^+ \longrightarrow Pheo_{red.} + Mg^{+2}$

5) $Pheo_{red.}$ + oxidant (but not safranine T) $\longrightarrow$ Pheo + reduced oxidant

6) $Pheo + AH^- \xrightarrow{h\nu} Pheo_{red.}$

Step 1) represents the dissociation of ascorbic acid in the presence of H_2O and the formation of the effective

electron donor, the ascorbate anion. Step 2), probably a complex reaction (to be described in a later paper), represents the photoreduction, the product of which is pink reduced chlorophyll ($Chl_{red.}$). Reduced chlorophyll can be oxidized by oxidants such as safranine T, regenerating chlorophyll (step 3), or it can react with hydrogen ions to form blue-gray reduced pheophytin ($Pheo_{red.}$) by step 4). Reduced pheophytin can be oxidized—though not by safranine T—to yield pheophytin (step 5). Step 6) represents the photoreduction of pheophytin yielding the blue-gray reduced pheophytin. The inability of reduced pheophytin to reduce safranine T suggests that it is a weaker reductant than pink reduced chlorophyll.

The quantum yield for the photoreduction of chlorophyll has not been previously reported. Under favorable conditions—30 % aqueous pyridine and ascorbic acid (0.10 M), the calculated value of the yield was about 5 %; since the possible sources of error in the measurements of the quantum yield would tend to give a low value; the actual yield could be higher—perhaps by as much as a factor of 2. The value of the yield, although not exceptionally large, indicates that under the conditions investigated, a significant fraction of the excitations of chlorophyll resulted in reduction. Thus, it is plausible that the photoreduction could be the primary process of photosynthesis.

Summary

1. The existence of the photoreduction of chlorophyll in aqueous pyridine solutions, in the presence of ascorbic acid is confirmed. The primary product is pink reduced chlorophyll as has been previously reported.

2. Water is required for the photoreduction, apparently, to dissociate ascorbic acid, thus forming the effective electron donor, the ascorbate anion.

3. Pink reduced chlorophyll, the 1st observed product of the photoreaction, can be oxidized by safranine T, presumably regenerating chlorophyll or, it can be acted upon by hydrogen ions to form blue-gray reduced pheophytin.

4. Reduced pheophytin generates pheophytin in the dark. Reduced pheophytin can also be formed directly by the photoreduction of pheophytin, a reaction which is completely reversible.

5. The quantum yield for the photoreduction of chlorophyll is dependent upon the water content; in 30 % aqueous pyridine with 0.10 M ascorbic acid, it is 5 % or more.

The author would like to thank Dr. Eugene Rabinowitch under whose guidance this work was performed.

In addition, the writer wishes to acknowledge a great indebtedness to Robert Emerson, whose presentation of the Stephen Hales Award Address in Columbus in 1950 first attracted the writer into the field of photosynthesis. In following years, the writer had the good fortune to be a student in Dr. Emerson's laboratory. Dr. Emerson's giftedness at teaching, his care and completeness in experimenting, his knowledge of fine tools and techniques, and his compassion and friendliness provide a standard of greatness. To have associated with and to have learned from a person of such greatness was the most important part of the writer's education.

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PHOTOREDUCTION AND PHOTOOXIDATION OF CYTOCHROME C BY SPINACH CHLOROPLAST PREPARATIONS[1,2]

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The photoreduction of cytochrome c by spinach chloroplasts has been noted by several investigators (12, 20, 25). In a preliminary report, we have presented evidence that aqueous digitonin extracts of spinach chloroplasts contain a cytochrome c photooxidase (22) which appears to be similar to the photooxidase present in extracts of photosynthetic bacteria studied by Kamen and Vernon (13, 14, 32). The present paper describes the behavior toward cytochrome c of whole chloroplasts prepared in hypertonic salt solution, and outlines the experiments which led to the demonstration of their cytochrome c-photooxidase activity.

MATERIALS AND METHODS

CYTOCHROME: The cytochrome c used in these studies was a preparation from horse heart obtained from the Sigma Chemical Co. Reduced cytochrome c was prepared by the method of Margoliash (18), or, alternatively, solutions of oxidized cytochrome c were reduced by addition of stoichiometric amounts of $Na_2S_2O_4$. In both cases, salts were removed by dialysis against H_2O. There was no difference between the behavior of these 2 reduced cytochrome preparations in the various enzyme tests used. All cytochrome solutions were analyzed prior to use for both oxidized and reduced cytochrome by measurement of optical density at 550 mμ before and after oxidation by ferricyanide and reduction by $Na_2S_2O_4$ (24).

DIGITONIN: Digitonin was obtained from Fisher Scientific Co., from National Biochemical Corp., and from Nutritional Biochemicals Co. It was recrystallized from ethanol. Although the various samples used exhibited different solubility characteristics, they all gave similar results in the experiments here described.

CHLOROPLAST SUSPENSION: Spinach was bought at the local markets. Suspensions of washed chloroplasts were prepared by differential centrifugation of ground spinach leaves in 0.35 M NaCl according to the procedure described by Arnon et al (2), with the modification that the NaCl solution was 10^{-4} M in disodium ethylenediamine tetraacetate (disodium Versenate), and the chloroplasts were washed twice. Chlorophyll was determined by the method of Arnon (1). Chloroplast suspensions were made up in the NaCl-Versene medium to contain about 1 mg of chlorophyll per ml and were stored in this form at 4° C. Precautions were taken to avoid unnecessary exposure to light, but no effort was made to exclude light completely. Examination of the suspension by light microscopy showed no detectable cell fragments or grana, but the apparently intact chloroplasts had an abnormal, vacuolated appearance.

PROCEDURE FOR ILLUMINATING SUSPENSION AND MEASURING REACTIONS: Measurement of light absorption was made with a Beckman DU spectrophotometer in cells of 1.00 cm light path, against water as a blank, with the slit width knob set at 0.04 mm. An increase in O.D. (optical density, i.e., log Io/I, where Io is incident light and I is emergent light) of 1 unit at 550 mμ corresponded to the reduction of 0.15 micromoles (μM) of ferricytochrome c in a volume of 3.0 ml. The samples were illuminated by a beam of white light focussed from above into the 2nd position of the cuvette carrier of the Beckman spectrophotometer. The portion of the cuvette which extended above the cuvette holder was shielded so that no scattered light from the light beam illuminating the reaction mixture could reach the photocell of the instrument. The intensity of the light at the surface of the liquid was about 40 ft-c. For the determination of O.D. the cell was pulled out of the light beam into the 1st position (i.e., in front of the photocell) for a period of 4 to 5 seconds, which sufficed to take a reading. The temperature was maintained at 20° ± 0.2° C.

RESULTS

PHOTOREDUCTION AND DARK OXIDATION OF CYTOCHROME c: Suspensions of washed chloroplasts, prepared as described above, caused a photoreduction of added cytochrome c in the light, and an oxidation of reduced cytochrome c in the dark. The photoreduction was conveniently demonstrated in dilute phosphate buffer of pH 7.0, containing about 0.06 μM of oxidized cytochrome c in a final volume of 3.0 ml. This solution was allowed to come to temperature equilibrium, the optical density at 550 mμ was measured, and then a suitable aliquot (e.g., 0.05 ml) of the chloroplast suspension was stirred into the reaction mixture under conditions of minimum illumination, without removing the cuvette from the instrument. A series of readings were taken in the dark at 1-minute intervals, to determine the contribution of the chloroplast suspension to the optical density at 550 mμ, and to ascertain whether any changes oc-

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[2] This investigation has been aided by grants from the American Cancer Society, from the National Science Foundation, and from the Wallace C. and Clara A. Abbott Memorial Fund in the University of Chicago.

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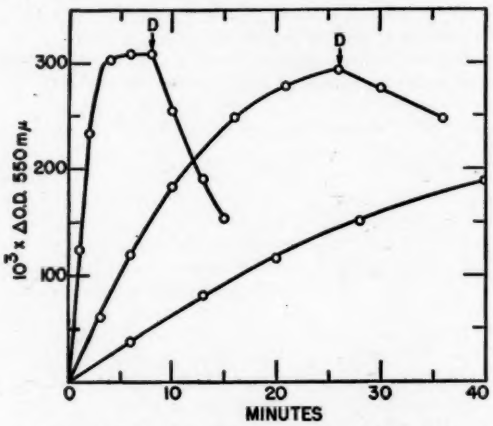
curred due to swelling or settling of the chloroplast suspension, or to any reduction of cytochrome c in the dark. No dark reduction of the cytochrome c was ever observed. Changes associated with swelling or settling were estimated to be no greater than 10 % of the changes measured, provided the amount of chloroplasts employed was less than that corresponding to 0.1 mg chlorophyll.

A typical experiment illustrating the photoreduction and the dark oxidation of cytochrome c in the presence of 3 different amounts of chloroplast suspension is shown in figure 1. The photoreduction stopped before the cytochrome was completely reduced. When the light was switched off (point D on the curves), the dark oxidation began, as shown by the decrease in optical density. Within the concentration range of chlorophyll employed in this experiment, the initial velocity of the photoreduction was approximately proportional to the amount of chlorophyll added. As higher concentrations were used, the reaction became too rapid for accurate measurement, light became limiting, and difficulty with settling of the suspension was likely to be encountered.

In the course of the investigation, rate measurements of photoreduction and dark oxidation were carried out with several hundred different chloroplast preparations. Approximately fifty were made by the same procedure, as described in the preceding section. With freshly prepared chloroplasts, the rates of photoreduction ranged from 15 to 63 μM of cytochrome c reduced per hour per mg chlorophyll. The chloroplast suspensions lost their activity slowly over a period of several days storage at 0° C. During the first few hours of storage, however, some of the preparations actually gained activity for photoreduction of cytochrome, but this rise was not always observed.

The dark oxidase activity of different chloroplast suspensions fluctuated even more than the capacity for photoreduction, and was not clearly correlated with the photoreduction. The kinetics of the 2 reactions were quite different. The photoreduction was saturated by relatively low concentrations of cytochrome so that the initial reaction was almost linear with time, but the dark oxidase was more sensitive to the concentration of reduced cytochrome, showing kinetics which sometimes were almost second order with respect to cytochrome concentration. This was not always the case, however. In general, the initial rate of the dark oxidation, when measured as shown in the experiments of figure 1, was about one third or one fourth the initial rate of photoreduction. At times, it was only one tenth as great, and more rarely, it was as fast as the photoreduction. When the dark oxidase activity was relatively high, the cycle of change from almost completely reduced to almost completely oxidized cytochrome could be repeated by alternating appropriate periods of light and dark up to 10 times. The rates gradually diminished, since the chloroplasts gradually lost their activity under the experimental conditions. Since the dark oxidase lost activity more rapidly than the photoreduction on storage at 0° C, suspensions kept in the cold for 1 or 2 days exhibited a smaller ratio of dark oxidation to photoreduction than fresh suspensions.

Effects of Inhibitors: Antimycin A, cyanide, and azide had no appreciable effect on the photoreduc-



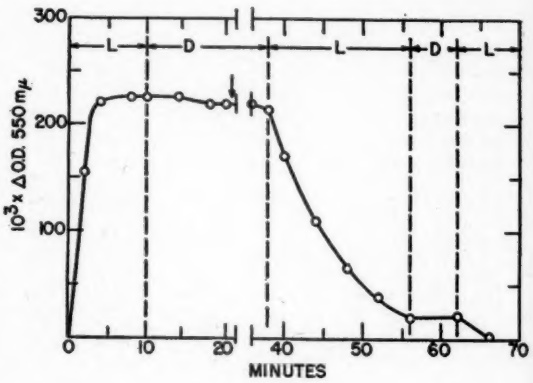


Fig. 1 (*left*) Photoreduction and dark oxidation of cytochrome c. Reaction mixtures contained 40 μM of potassium phosphate buffer of pH 7.0, 0.06 μM of cytochrome c, and water to make a final volume of 3.0 ml. The lower, middle, and upper curves were obtained with chloroplasts containing 0.0044, 0.0176, and 0.0705 mg of chlorophyll, respectively. Light was turned on at time 0, turned off at arrow D, for each experiment.

Fig. 2 (*right*). Unmasking of photooxidase by digitonin. Reaction mixture contained 40 μM of phosphate buffer of pH 7.0, chloroplasts containing 0.1 mg of chlorophyll, 0.3 μM of KCN, and 0.056 μM of cytochrome c. L: light; D: dark. At the vertical arrow, 0.2 ml of aqueous 1 % digitonin were added. O.D. readings corrected as described in text.

tion of cytochrome c, but the dark oxidase was inhibited almost completely by cyanide and by azide. Some representative data showing the effect of these 2 compounds are assembled in table I, which also shows that the photoreduction is inhibited by increasing the concentration of phosphate buffer. All the rate measurements listed in table I were carried out with the same chloroplast suspension, which had been stored for several hours. The effects observed were reproducible with other suspensions, except for the effect of phosphate on the dark oxidase. In the particular experiment shown in table I, the higher concentration of phosphate caused a moderate stimulation of the dark oxidase, but with other chloroplast preparations, the higher concentration of phosphate sometimes caused a small inhibition of the dark oxidase.

Table I also shows the percent of cytochrome in the reduced form when the steady-state level was reached, i.e., when the optical density at 550 mμ showed no further change on continued illumination. As already mentioned, the photoreduction of cytochrome did not reach completion. The level of reduction of the cytochrome was calculated from the observed change in optical density and the known amount of cytochrome added, and this calculation was corroborated by addition of sodium hydrosulfite at the end of the experiments with measurement of the attendant increase in optical density which accompanied the complete reduction of the cytochrome.

In the experiments with added cyanide, precautions were required in the determination of the steady-state level. The oxidized form of cytochrome c forms a complex with cyanide, and this cyanferricytochrome c complex is not photoreduced by the chloroplasts. In this respect, the photoreduction of cytochrome c is completely analogous to the reduction of cytochrome c by succinate in the presence of preparations from animal tissues. Horecker and Kornberg (9) have studied the cytochrome c-cyanide complex in some detail, using the extent of reduction by succinate as a measure of the amount of uncomplexed cytochrome present. It is possible to use the photoreduction of cytochrome by chloroplasts in the same fashion. Exposure of the cytochrome to high concentrations of cyanide for a sufficient time produces total complexing and consequently apparent inhibition of photoreduction. When 10^{-4} M cyanide is added just prior to illumination, however, only a negligible amount of the complex is formed.

TABLE I

EFFECT OF PHOSPHATE, AZIDE AND CYANIDE ON THE CHLOROPLAST-CYTOCHROME c REACTIONS

PHOSPHATE, pH 7.0 μM	OTHER ADDITIONS μM	PHOTOREDUCTION *	DARK OXIDATION *	STEADY STATE LEVEL ** %
40	...	9.1	1.9	90
200	...	4.4	2.5	51
200	0.3 KCN	4.1	0.3	79
200	0.3 NaN_3	3.9	1.1	70
200	15 NaN_3	4.4	0.2	71

* Initial rate, micromoles per hour per mg chlorophyll. The reaction mixtures contained 0.056 μM of cytochrome c and chloroplasts containing 0.05 mg of chlorophyll, plus other additions as indicated, in a volume of 3 ml.

** Percent cytochrome c in reduced form.

The steady-state level of cytochrome c reduction was expected, at first approach, to reflect a balanced state determined both by the rate of the photoreduction and by the rate of the dark oxidation. The data in table I indicate a tendency in this direction. Thus the increase in phosphate concentration which inhibited the photoreduction also caused an appreciable drop in the percent of cytochrome c present in the reduced form at the steady-state level. Efforts to ascertain whether the measured rate of photoreduction and dark oxidation could account completely for a particular steady-state level were not successful because of the difficulty of handling the kinetics, but the observations suggested that the rate of the dark oxidase did not always give an accurate measure of the rate of cytochrome oxidation during illumination.

CYTOCHROME c PHOTOOXIDASE: The presence of a cytochrome c photooxidase in spinach chloroplasts could be demonstrated very clearly by inhibiting the photoreduction with digitonin. The effect of the reagent is illustrated in figure 2. In this experiment, the photoreduction of cytochrome c was carried out in the presence of cyanide till a steady-state level was reached. Switching off the light resulted in no change in the optical density, since the dark oxidase was almost completely inhibited by the cyanide. At 21 minutes (arrow), 0.2 ml of 1 % digitonin (i.e., 2 mg) was added, with minimum exposure to light. There was a slow change in optical density in the dark which was associated with the action of the digitonin on the chloroplasts, and not with any appreciable change in the oxidation-reduction state of cytochrome c. The amount of this change was determined in separate experiments, and the optical density readings were corrected for this digitonin effect, and for dilution. After about 16 minutes, when the optical density readings had again reached an almost constant value, the light was switched on. There was a steady decrease in optical density in the light, as shown in the graph. If the light was switched off, the change in optical density ceased. When illumination was resumed, the oxidation also resumed, with no lag period. The cytochrome c was eventually completely reoxidized. It could be re-reduced by addition of a stoichiometric amount of sodium hydrosulfite, and the photooxidation could be repeated.

REDUCTION BY SUCCINATE: Brummond and Burris (4) have reported that the succinic dehydrogenase activity of green leaves is soluble and unstable. They measured this enzyme reaction by coupling it with N-methyl phenazine sulfate. In the present experiments, fresh chloroplast suspensions with added cyanide to inhibit the dark oxidase, were shown to catalyze the reduction of cytochrome c in the dark by

added succinate. This activity was very unstable relative to the other activities measured, and disappeared on storage of the chloroplasts for several hours. A typical measurement with 0.3 μM of KCN and 10 μM of succinate under the other conditions described in the legend for figure 1 gave an O.D. increase at 550 mμ of 0.070, equivalent to 0.01 μM cytochrome c reduced per minute per mg chlorophyll. This reduction of cytochrome by succinate was not inhibited by antimycin A or by the addition of digitonin. Thus, chloroplasts in the presence of digitonin, cyanide, and succinate, would reduce cytochrome c in the dark and oxidize it in the light, in direct contrast to the behavior of unsupplemented chloroplasts, which reduced cytochrome c in the light and oxidized it in the dark.

Solubilization and Properties of Cytochrome c Photooxidase: Digitonin has been used previously to prepare chlorophyll-lipid-protein complexes which are not sedimentable by ordinary centrifugation and have been called "chloroplastin" (15, 29, 30, 31, 33). The following experiments showed that such solutions contain the cytochrome c photooxidase activity. Twice washed chloroplasts from 200 g of spinach leaves were extracted at 0° C with 3 successive 10-ml portions of 1 % digitonin, the 1st for 30 minutes, the 2nd for 40 minutes, the 3rd for 2.5 hours. Each successive extract was separated from the residue by centrifugation at 75,000 × G for 30 minutes. These extracts were labelled A, B and C, in the order of their preparation. When such extracts were centrifuged for 30 minutes at 144,000 × G in a Spinco preparative ultracentrifuge, relatively trivial amounts of sediment were obtained, and the activity of the supernatant solution remained unchanged. It was possible, however, to draw off a turbid yellow supernatant from the top few millimeters, just above the clear green boundary which had appeared during the centrifugation. This yellow supernatant was free from chlorophyll, and contained no trace of photooxidase activity. This implied that "chloroplastin" was necessary for the photooxidase activity. Nevertheless, the activity of the successive extracts was not proportional to their chlorophyll content, as shown by the data given in table II. Included in this table are the results of experiments showing that the photooxidase was completely inactivated by heat, and that the various heated extracts all contained an activator for the photooxidase.

Table II

Chlorophyll Content and Photooxidase Activity of Successive Digitonin Extracts

Extract	Heated extract *	Chlorophyll ** mg/ml	Photooxidase activity †
A	...	0.096	5.1
...	A		0
B	...	0.216	3.5
...	B		0
B	A		9.4
B	B		7.3
B	C		6.3
C	...	0.074	2.4
...	C		0

* Held at 90° C for 5 minutes. 0.2 ml used per reaction mixture.

** Chlorophyll content of unheated extract.

† Micromoles cytochrome c oxidized per hour per mg chlorophyll of the unheated extract. Assay mixtures contained 300 μM of phosphate buffer of pH 7.0, 0.03 μM of reduced cytochrome and other additions as indicated, in a volume of 3 ml. The unheated extract was used in sufficient amount to give approximately 0.05 mg chlorophyll.

The assay of cytochrome c photooxidase was based on the fact that cyanide inhibits the dark oxidase activity of the chloroplastin extracts, but not the photooxidase. Curve 1 of figure 3 shows the dark and light oxidation of ferrocytochrome c observed in the presence of cyanide. In the absence of cyanide (curve 2), the dark oxidation was more rapid, and the change in rate on illumination was not so striking. Examination of a large number of preparations showed that the ratio between the rate of the dark oxidation, measured in the absence of cyanide, to the rate of the photooxidation, measured in the presence of cyanide, varied considerably. The rate of oxidation of cytochrome c in the light in the absence of cyanide was either equal to or slightly greater than the rate in the light in the presence of cyanide. The data suggested that the 2 reactions were independent, at least in part, though they might have a common "bottleneck."

Under the conditions used in the experiment shown in figure 3, the initial rate of photooxidation in the presence of cyanide was almost proportional to the amount of extract added, provided the concentration of reagents was kept constant and provided the rates were not faster than $\triangle$O.D. = 0.020/min. (The figures given in table II were measured in this way.) This apparent proportionality was due to 2 opposing effects which tended to balance each other. The photooxidase activity of the digitonin extracts required 2 heat-labile components, present in the extracts. However, as larger amounts of chlorophyll were employed, light became more limiting, so the dependence of rate on the square of the concentration of extract was not evident except at very low concentrations. Cytochrome c photooxidase was strongly stimulated by phosphate and other salts, an effect which will be described in another paper.

The photooxidase was not affected by freezing and could be stored for months at −15° C. On storage at 4° C there was a progressive and somewhat variable inactivation which amounted to as much as 50 % in 4 days. The activity was stable to dialysis against dilute phosphate buffer of pH 7.0. Both the dark oxidase and the photooxidase were heat-inactivated. There was no precipitate formed when digitonin extract B was heated, so this extract was generally used for testing effects of heat.

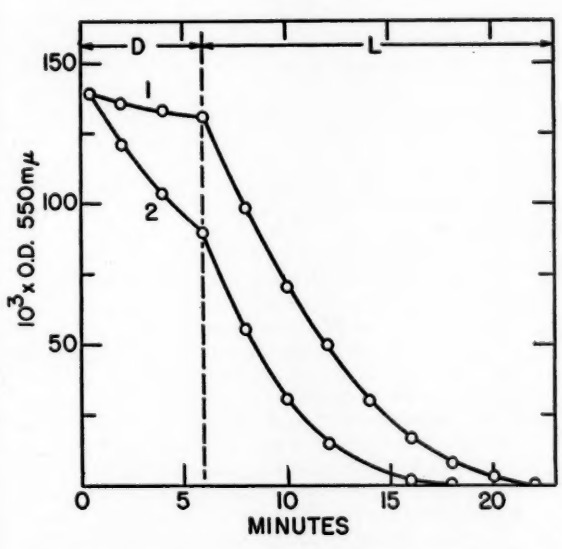
Figure 4 shows representative data to illustrate how some of the conclusions cited were reached.

Curve 1 shows that there was no significant change in optical density when reduced cytochrome c was incubated with 0.2 ml of heated digitonin extract. The 1st 6 minutes of incubation were in the dark. Then the light was turned on at 6 minutes (broken line). At 28 minutes the light was turned off and 0.1 ml of unheated extract was added (arrow D). The change due to the dark oxidase was noted, and then the light wa switched on at arrow L. The rate of photooxidation is seen to be faster than the activity displayed by 0.2 ml of unheated extract alone (curve 3). Curve 2 shows the activity of 0.1 ml of unheated extract. Curves 2 and 3 show that the rate of photooxidation with 0.2 ml of extract is about twice as great as the rate with 0.1 ml of extract. Addition of 2 mg of digitonin had no effect on the reaction rates observed.

The heat instability of the photooxidase implied that the reaction was enzymatic. To corroborate this conclusion, 0.2 ml of a fresh acetone extract of the chloroplasts, containing an amount of chlorophyll comparable to that used in the photooxidase test, was added to the cytochrome c-phosphate buffer test system. No activity whatever was observed. Acetone in the quantity employed did not inhibit the photooxidase activity of the digitonin extracts.

The choice of pH 7.0 for the measurement of the photooxidase represented a compromise between the apparent optima for the photooxidase activity and for the cyanide inhibition of the dark oxidase. The latter was completely inhibited by cyanide (10^{-4} M) at pH 7.5 and 8.0, but was only partially inhibited at lower pH values, possibly because of the volatilization of HCN. A pH of 6.3 appeared to be more nearly optimal for the photooxidase, giving a 20 % increase in rate over that observed at pH 7.0. However, the effect of pH on the dark oxidase in the presence of cyanide was even greater, the rate at pH 6.3 being twice that at 7.0. The rates of the photooxidase at pH 7.5 and 8.0 were about two thirds and one third respectively of the rate at 7.0. In the range pH 5.3 to 5.9, turbidity developed and the photooxidase was inactivated.

Cytochrome c photooxidase requires the presence of oxygen, as shown in figure 5. Cytochrome c



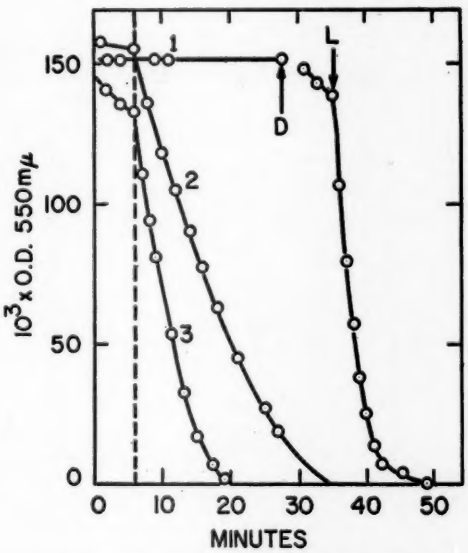


FIG. 3 (*left*). Demonstration and assay of cytochrome c photooxidase. Reaction mixtures contained 80 μM of phosphate buffer of pH 7.0, extract containing 0.04 mg of chlorophyll, and 0.06 μM of cytochrome c, of which 0.02 μM were in the reduced form, in a total volume of 3.0 ml. Curve 1: no KCN; curve 2: 0.3 μM of KCN. D: dark; L: light. Manipulations were carried out as described for the experiments of figure 1. O.D. readings corrected for optical density of extract and oxidized cytochrome c.

FIG. 4 (*right*). Photooxidase of dialyzed extract. Experiments were carried out with digitonin extract B, containing 0.18 mg of chlorophyll per ml, dialyzed for 16 hours at 4° C against 0.01 M phosphate buffer of pH 7.0. All reaction mixtures contained 40 μM of phosphate buffer of pH 7.0; 0.02 μM of reduced cytochrome c, 0.3 μM of KCN, and other additions as indicated, to a total volume of 3.0 ml. All reactions were started in the dark, and light was turned on at 6 minutes. Curve 1: 0.2 ml of extract B which had been held at 90° C for 5 minutes was added at time 0; at D, light was turned off, and 0.1 ml of unheated extract B was added; at L, light was turned on. Curve 2: 0.1 ml of unheated extract B added at time 0. Curve 3: 0.2 ml of unheated extract B added at time 0. All O.D. readings corrected for optical density of extract and oxidized cytochrome c.

photooxidase was not affected by added catalase or TPN, and was completely inactive toward 2,6-dichlorobenzenone-indophenol. In the latter test, 0.05 μM of dye were used, and optical density readings were taken at 620 mμ. The dye was neither reduced nor oxidized either in the light or in the dark in the presence of the cytochrome c photooxidase preparations.

Discussion

Chloroplasts prepared by the method of Arnon et al (2) resemble those studied by Jagendorf (12) in that photoreduction of cytochrome c can readily be demonstrated in the absence of respiratory inhibitors. With the chloroplast suspensions studied by Rosenburg and Ducet (25) and by Mehler (20), it was necessary to inhibit the oxidase by cyanide in order to demonstrate that cytochrome c could function as a Hill reagent. McClendon (19) and Jagendorf and Wildman (11, 12) have described chloroplast preparations devoid of cytochrome oxidase, and are of the opinion that this enzyme activity represents a "contamination" of chloroplasts with other particulate matter from the leaf. On the other hand, Sissakian and his co-workers (27, 28) favor the view that cytochrome oxidase activity is indigenous to the chloroplasts. We have been impressed in the present experiments with the great variability of the (dark) cytochrome oxidase content of the various chloroplast preparations, and with the occasional very high values observed for the activity of this enzyme. Furthermore, on the one occasion when this was deliberately tested, the dark oxidase activity of the chloroplasts was enhanced by repeated washing with 0.35 M NaCl solution. The experiments of James and Das (10) have shown clearly that Krebs cycle enzymes with associated respiratory enzymes are separable from chloroplasts in a mitochondrial fraction. It is quite possible that the chloroplasts prepared according to Arnon et al (2) are contaminated with mitochondria to some extent, but it is unlikely that any such contamination represents a very high proportion of the bulk of the preparation. (We have not been able to see any evidence of contamination under the light microscope, and examination of the preparations with the electron microscope revealed only a small proportion of debris which did not appear to be of chloroplastic origin.) We do not regard the fact that chloroplasts can be prepared so as to be relatively free of cytochrome oxidase as evidence that the oxidase has been removed rather than inactivated. Since the cytochrome oxidase activity of the chloroplasts was sometimes quite high, the implication is that the cytochrome oxidase activity of the contaminating mitochondria must be very high indeed. James and Das (10) report that mitochondria from broad bean leaves have cytochrome c oxidase activity amounting to 560 μl of O_2 per hour per mg of N. Values approaching this level were sometimes observed with the whole chloroplasts.

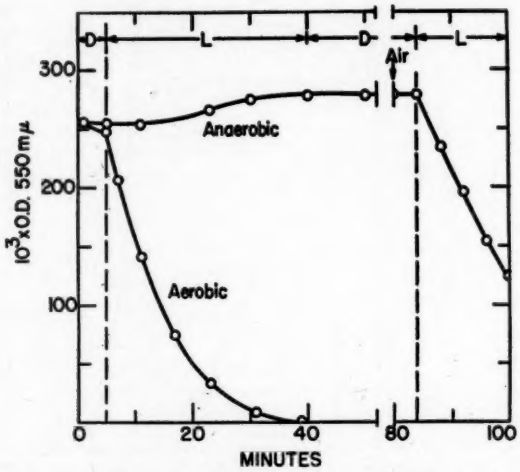


Fig. 5. Dependence of photooxidase on O_2. Reaction mixtures contained 80 μM of phosphate buffer of pH 7.0, 0.3 μM of KCN, extract containing 0.021 mg of chlorophyll, and 0.108 μM of cytochrome c of which 0.038 μM were in the reduced form, in a total volume of 3.0 ml. O.D. readings corrected for chlorophyll and oxidized cytochrome. D: dark; L: light. Both reaction mixtures were illuminated with 2,000 ft-c outside of the Beckman spectrophotometer for carefully timed intervals. O.D. was measured by inserting the cuvette in the cell holder and quickly taking a reading. The anaerobic incubation was carried out in a cuvette fitted with a hollow stopper and side arm for evacuation. The chlorophyll-containing solution was added from the stopper after air had been removed by thorough evacuation.

The above remarks refer to the dark cytochrome oxidase activity of the whole chloroplasts. For the cytochrome c photooxidase, there can be no question regarding its chloroplastic origin, since, as will be shown in a subsequent paper, a chlorophyll-lipid-protein complex is one of the components essential for the photooxidation reaction. The effect of digitonin on the photooxidase is regarded as an unmasking. When the chloroplasts are treated with digitonin they undergo extensive fragmentation, and lose their capacity to photoreduce cytochrome c. This permits their photooxidase activity to be demonstrated directly. The mechanism of the inhibitory effect of digitonin on the photoreducing capacity of the chloroplasts is of some interest. When plant mitochondria are treated with digitonin, their capacity for causing the dark oxidation of cytochrome c is enhanced (26). One might suppose that the photooxidase activity is enhanced by digitonin in a similar manner, but it is unlikely that the entire effect of digitonin on chloroplasts can be explained in this way. The behavior of the preparations suggests a severe inhibition of the photoreducing power of the system. It should be noted, however, that the action of the digitonin is gradual, and to some extent reversible. Chloroplast preparations which appear to have been completely

inhibited insofar as photoreduction of cytochrome c is concerned, can be partially reactivated by washing out the digitonin. When chloroplasts are extracted with aqueous digitonin, considerable green sediment always remains after centrifugation. Only a relatively small proportion of the total chlorophyll is solubilized to a point where it is not very readily sedimented at 144,000 × G. From chloroplasts suspended in aqueous digitonin one can in fact obtain, by suitable differential centrifugation, green fragments smaller than grana and capable of photophosphorylation as well as of photoreduction (5). The digitonin extracts described in the present paper do not yield such fragments because these have been removed by the high-speed centrifugation. These facts are of interest in connection with the recent report of Eversole and Wolken (8) who have described a number of photochemical activities in "chloroplastin" preparations.

Davenport, Hill and Whatley (6, 7) have described a protein factor present in leaf extracts necessary for the photoreduction of methemoglobin and cytochrome c by chloroplast suspensions prepared in a different way from those used in the present experiments. A possible explanation for the effect of digitonin in our experiments is that it causes the release or inhibition of this factor, which is presumably present in sufficient amount in the intact chloroplasts to permit photoreduction of cytochrome c without added activator. The digitonin may act partially in this manner, though it probably has a more deep-seated additional effect on the photolysis reaction, since the photoreduction of Hill reagents other than cytochrome c is also inhibited by digitonin treatment. The digitonin apparently inhibits the O_2-evolving system. The possibility that the cytochrome c photooxidase is an artifact created by the digitonin has been carefully considered. We have already pointed out (22) that the cytochrome c photooxidase here described is different from the non-enzymatic photochemical reactions studied by Krasnovsky and his associates (16, 17). This difference is even more clearly brought out by the demonstration that the enzymatic photooxidase can be fractionated into 2 heat-labile components, one containing chlorophyll, and one not (21, 23).

We view the cytochrome c photooxidase as a component of the intact chloroplasts, partly because of the kinetic measurements which led us to suspect the presence of the photooxidase before we found a procedure for unmasking it. It is of interest that the data obtained with heavy oxygen by Brown and Good (3) seems entirely compatible with the presence of a cytochrome c photooxidase in their preparations. Their data with cyanide-poisoned chlorella suggests, in fact, that a cyanide-insensitive photooxidase is operating in these algae. Perhaps the photooxidase described in the present paper can account in part for that component of the oxygen absorption which does not appear to involve reduction of O_2 to H_2O_2.

Summary

This paper describes the behavior toward cytochrome c of spinach chloroplasts prepared in a suspending medium of 0.35 M NaCl. The chloroplasts cause a photoreduction of ferricytochrome c, and a dark oxidation of ferrocytochrome c. They also contain a cytochrome c photooxidase, which can be clearly demonstrated after the photoreduction reaction has been inhibited with digitonin. The enzyme or enzyme complex causing the photooxidation of cytochrome c can be solubilized by treatment with digitonin. The cytochrome c photooxidase is not inhibited by cyanide and may thus be distinguished from the cyanide-sensitive dark oxidase.

The authors are indebted to Drs. Hans Gaffron and Norman Bishop for stimulating discussion.

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FRACTIONATION AND PURIFICATION OF CYTOCHROME C PHOTOOXIDASE OF SPINACH [1, 2]

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The preceding paper described evidence for the presence of a cytochrome c photooxidase activity in digitonin extracts prepared from washed spinach chloroplasts (3). The present paper describes how this photooxidase may be fractionated into 2 components, designated Factor 1 and Factor 2, both of which are necessary for enzyme activity. Factor 2 is soluble, and a procedure is described for its purification.

MATERIALS AND METHODS

MEASUREMENT OF CYTOCHROME c PHOTOOXIDASE: The assay previously described (3) depended on the use of cyanide to inhibit the dark cytochrome oxidase, and measurement of the rate of photooxidation of cytochrome c by following the decrease in absorption at 550 mμ. This assay procedure was used during the experiments leading to the initial fractionation of the enzyme components. Protein was determined according to Lowry et al (2).

PREPARATION OF DIGITONIN EXTRACTS: The medium used to prepare the washed chloroplasts contained 0.35 M NaCl and 10^{-4} M disodium ethylenediaminetetraacetate. This is referred to as the NaCl-Versene solution.

Fresh washed spinach leaves were drained well, and stems were removed. Five 200-g lots of leaves were conveniently handled at one time. Each lot was chopped fine and transferred to a Waring blendor with an equal volume (200 ml) of ice-cold NaCl-Versene solution. The material was blended for 4 or 5 half-minute periods with the variac set at 90 v. In the intervals, the suspension was stirred to resuspend the solids. The homogenate was strained through 2 layers of

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cheese cloth and transferred to 250 ml centrifuge cups in the G/1-R head of a refrigerated Servall centrifuge, with the temperature control set at 0° C. The rotor was allowed to accelerate to 1000 rpm, and the current was turned off. The dark green supernatant suspension was decanted from the sediment, which was discarded. The supernatant was recentrifuged at 2,500 × G (3,500 rpm) for 10 minutes. The green precipitate, which consisted mainly of intact chloroplasts, was separated by decantation, resuspended in sufficient NaCl-Versene solution to restore the original volume, and centrifuged again as previously described. This washing was repeated. The washed chloroplasts were then extracted with 1 % aqueous digitonin solution (0.05 ml per g of fresh leaf). The green sediment was dispersed in the digitonin solution, and transferred to 50 ml centrifuge tubes. In this process, the material was allowed to come to room temperature, and kept at this temperature with occasional stirring, for about 20 minutes, then centrifuged for 30 minutes at top speed (25,000 × G) at 0° C in the SS-1 head of the Servall centrifuge. The clear green supernatant solution was carefully decanted and could be stored overnight in the ice box, or for longer periods at −15° C. The green sediment was sometimes re-extracted with 1 % aqueous digitonin solution, and the 2 successive extracts were combined for the fractionation.

Fractionation of Digitonin Extract: The following fractionation procedure was found to be consistently successful in separating the cytochrome c photooxidase activity of the digitonin extracts into 2 fractions, inactive separately, and active in combination. In outline, the method consisted of precipitation of an insoluble, chlorophyll-lipoprotein complex from the digitonin extracts by addition of ethanol. This gave 1 of the necessary components, Factor 1, which contained the chlorophyll. The 2nd component, Factor 2, was obtained from the supernatant by acetone fractionation.

The clear green chloroplast extract prepared with aqueous digitonin was cooled in an ice bath, and ethanol, pre-cooled to −15° C, was added dropwise, with vigorous stirring, until 0.3 ml of absolute ethanol had been added per ml of extract. After 15 minutes, the mixture was centrifuged for 20 minutes at 25,000 × G and 0° C. The bulk of the chlorophyll was sedimented by this procedure. The supernatant was decanted. The sediment was resuspended in 1 % aqueous digitonin solution (10 ml per kg fresh leaf). This preparation called Factor 1, could be stored at −15° C. Expressed in terms of chlorophyll, the yield of Factor 1, when 2 digitonin extractions were made, ranged from about 10 to 30 mg of chlorophyll per kg of fresh spinach leaf, with an average of about 25.

The supernatant from the ethanol precipitation contained Factor 2, which was separated as follows. Acetone pre-cooled to −15° C was added in the manner described for the previous ethanol addition. When 1 ml of acetone had been added per ml of supernatant, the solution was allowed to stand in the ice bath for 15 minutes, then centrifuged at 0° C, and 2,500 × G for 20 minutes in the G/1-R head of the Servall centrifuge. The precipitate contained any green pigment that had not been precipitated by the ethanol, and a considerable amount of yellow pigment. Much of this material was not water soluble. It contained a component which stimulated the photooxidation, but was largely heat-stable and was not further characterized. This first acetone precipitate was generally discarded.

After removal of the first acetone precipitate, a volume of acetone equal to twice the volume used in the previous step was added to the supernatant in the cold as previously described. The mixture was allowed to stand in the ice bath for 30 minutes, then centrifuged as previously described for 30 minutes. At this stage, the centrifuge bottles could be allowed to stand overnight in the ice box. If more precipitate appeared, the centrifugation was repeated. The supernatant was poured off and allowed to drain well. The precipitate, which contained Factor 2, was dissolved in 0.01 M phosphate buffer of pH 7.0 (5 or 10 ml per kg fresh leaf), and stored at −15° C.

When 2 digitonin extractions were employed, the protein content of the active precipitate of Factor 2 was about 15 to 20 mg per kg of fresh spinach. The solution of Factor 2 varied in color from pale brown or yellow to brown-green. On occasion, a bright blue fluorescence superimposed on a yellow color, appeared to be responsible for the greenish appearance. The intensity of pigmentation was variable in different preparations and had no demonstrable relationship to the activity.

One may depart rather widely from the procedure described above, and still obtain active preparations. The major effect of using 2 digitonin extractions rather than 1, is an increase in yield of variable magnitude. This increase is greater for Factor 1 than for Factor 2. Extensive inactivation occurs at the stage where Factor 1 is precipitated by ethanol if the temperature is not kept low, or if an excess of ethanol is added. Such inactivation is usually accompanied by the extraction of some of the chlorophyll into the aqueous ethanol phase. Once the chlorophyll is extracted from the lipoprotein complex, activity is lost completely.

Results and Discussion

Reconstitution of Photooxidase from Components: Figure 1 shows the evidence that cytochrome c photooxidase activity is dependent on both Factors 1 and 2. Factor 2 alone had no effect on the oxidation-reduction state of cytochrome c either in the light or in the dark, as shown by the top line. The next curve shows the effect of Factor 1 alone. During the 1st 4 minutes in the dark, there was a small increase in optical density, which was not due to reduction of cytochrome but to a change in the absorption due to Factor 1 itself. This material was added at 0 time in a small volume of 1 % digitonin solution. On dispersion in the water, a very fine suspension was

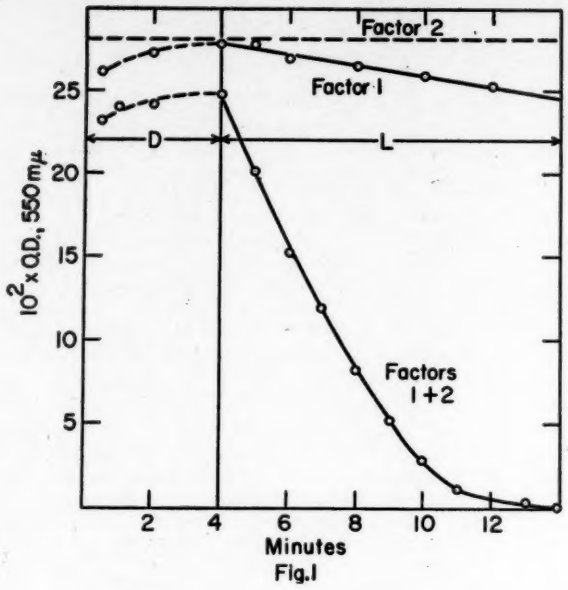

Fig. 1

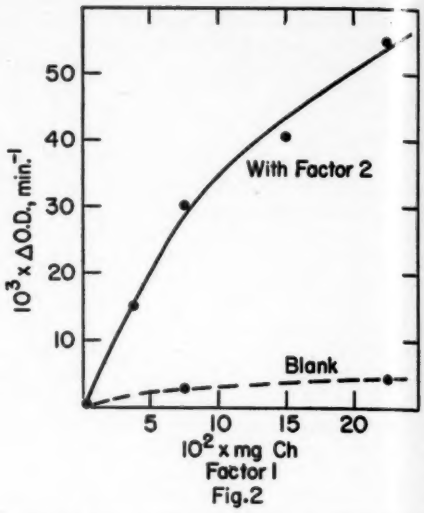

Fig. 2

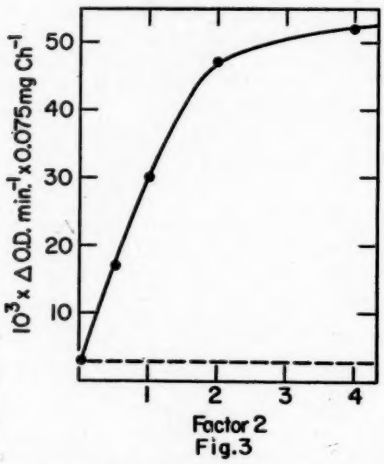

Fig. 3

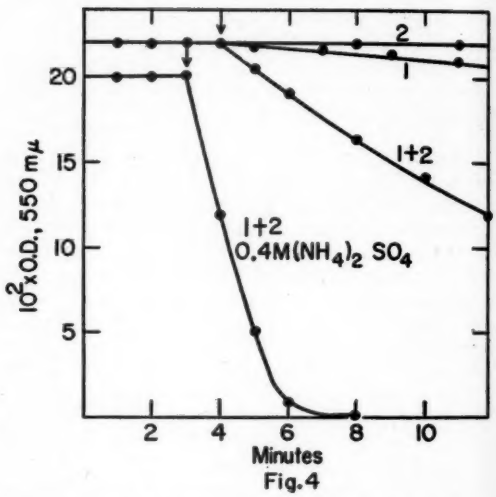

Fig. 4

FIG. 1. Dependence of cytochrome photooxidase on two components. The test system contained 300 μM of phosphate buffer of pH 7.0, 0.5 μM of KCN, and 0.038 μM of reduced cytochrome c in a final volume of 3.0 ml. D: dark; L: light. Factor 2 was added as 0.4 ml of the preparation described in the text. The preparation of Factor 1 described in the text was diluted with 1 % digitonin to contain 0.75 mg chlorophyll per ml. The reaction was started by addition of 0.1 ml of this solution to the reaction mixture in the manner previously described (3). Temperature = 20° C. Illumination, 40 ft-c. O.D. readings were corrected for the absorption by the oxidized cytochrome and by the final absorption of Factor 1 alone, and are proportional to the amount of reduced cytochrome present, except for the first few minutes in the dark.

FIG. 2. Dependence of cytochrome c photooxidase on the amount of Factor 1. Rates are expressed as Δ O.D. per minute observed in the test system described for figure 1. For the measurements made in the upper curve, 0.4 ml of Factor 2 was added. The amount of Factor 1 is given in terms of its chlorophyll content.

FIG. 3. Dependence of cytochrome c photooxidase on the amount of Factor 2. All measurements were made in the test system described for figure 1, with a constant amount of Factor 1, equivalent to 75 μg of chlorophyll in each reaction vessel. Figures along abscissa represent ml $\times$ 10 of the solution of Factor 2.

FIG. 4. Effect of $(NH_4)_2SO_4$ on the assay of cytochrome c photooxidase. Conditions and procedure are the same as for the experiments of figure 1, except as noted. The light was turned on at the arrows. Curve 1: optical density change with an amount of Factor 1 containing 38 μg of chlorophyll. Curve 2: optical density change with 0.1 ml of Factor 2. Curve 1 + 2: optical density change with Factors 1 and 2 combined. The lower curve was obtained in identical fashion as that above it, except that the reaction mixture was 0.4 M in $(NH_4)_2SO_4$.

formed. The particles were well suspended and did not settle during the time normally employed for measurements. There was always an increase in optical density, however, which ceased after 4 or 5 minutes. When the light was turned on, a slow photooxidation occurred in the presence of Factor 1 alone. The rate was small, however, compared to the rates observed if Factor 1 was added to a test solution which had been supplemented with Factor 2, as shown in the lower curve. The photooxidation continued until all of the reduced cytochrome c had been oxidized.

The slow rate of photooxidation observed with Factor 1 alone was due to the fact that this factor was separated as a bulky precipitate, and retained a little of the other factor. Methylene blue, or solutions of chlorophyll in organic solvents could not be substituted for Factor 1. Added catalase or triphosphopyridine nucleotide had no effect on the reaction.

The reaction rate of the photooxidase was dependent on the concentrations of both Factor 1 and Factor 2. In Figure 2 are shown the initial reaction rates observed with increasing amounts of Factor 1, the chlorophyll-containing component. The upper curve was obtained with a constant amount of Factor 2 and increasing amounts of Factor 1. The lower curve shows the rates observed with Factor 1 alone in the absence of Factor 2. The reaction rate was almost linearly proportional to the amount of Factor 1 at lower concentrations, but this linearity did not apply as increasing amounts of chlorophyll were used. As described in a later paper (1), light becomes increasingly limiting as higher amounts of Factor 1 are tested, and this is the reason for the downward bend of the curve in Figure 2. Figure 3 shows the dependence of the reaction rate on the concentration of Factor 2. With smaller amounts of Factor 2, the rate was almost linearly proportional to the amount added. Such a system could therefore be used to assay Factor 2, provided care was employed to use sufficiently small quantities to stay on the linear portion of the curve. The dashed line represents the blank value of the rate with Factor 1 alone, a figure which must be subtracted in the assay for Factor 2.

DARK OXIDASE OF FACTOR 1: The dark cytochrome oxidase activity of Factor 1 was tested in 0.05 M phosphate buffer of pH 7.0 by following the decrease in O.D. at 550 mμ. With 0.038 micromoles (μM) of reduced cytochrome c in 3 ml, a typical rate of oxidation observed was 1.25 μM of cytochrome c oxidized per minute (or 420 μl O_2 per hour) per mg of chlorophyll. The kinetics of the reaction insofar as they were examined, resembled those of cytochrome oxidase from animal tissues as described by Smith and Conrad (4). The rate was approximately first order with respect to reduced cytochrome c. Addition of Factor 2 in amounts up to 0.4 ml had no appreciable effect on the rate of this dark oxidation of cytochrome c.

COMPOSITION OF FACTOR 1: Six different samples of Factor 1 were analyzed for protein as well as for chlorophyll. The ratio of protein to chlorophyll was relatively constant, ranging from 5.2 to 6.7 mg protein per mg chlorophyll, with an average of about 6. Though the supernatant from Factor 1 contained a yellow lipid fraction, much yellow lipid was still retained in the Factor 1 fraction.

Considerable phosphorus was also present in Factor 1. One sample contained 1.7 μM of phosphorus per mg of chlorophyll. Of this total, 0.4 μM was inorganic orthophosphate, and 0.2 μM was acid soluble organic phosphorus. These 2 fractions were extracted into 0.2 *N* perchloric acid in 10 minutes at 0° C. The phospholipide phosphorus (extracted by a 3 : 1 mixture of ethanol : ether) amounted to 0.7 μM phosphate per mg chlorophyll. Almost all of the remaining phosphorus (0.4 μM per mg chlorophyll) was extracted in 20 minutes at 70° C by 0.5 *N* perchloric acid, and is presumably nucleic acid, as deduced from the absorption spectra of the extracts.

When all of the chlorophyll was removed from Factor 1 by extraction with aqueous acetone, a pinkish brown residue remained. If this residue was suspended in water and subjected to centrifugation, a layer of tomato-red pigment collected on top of the insoluble residue. This pigment could be scraped from the surface and dissolved in petroleum ether, with a change of the color to yellow. The yellow petroleum ether solution had absorption maxima at 448 mμ, 250 mμ and 340 mμ (listed in order of decreasing intensity). The brown insoluble residue contains chlorophyllase. Recombination of the residue with the chlorophyll dissolved in aqueous acetone results in the loss of the phytyl moiety from the chlorophyll. Prior to its extraction by acetone, the chlorophyll is not hydrolyzed by the chlorophyllase, even though the two are present together in the chlorophyll-lipoprotein complex [4].

ASSAY WITH AMMONIUM SULFATE: During a study of the use of ammonium sulfate to purify Factor 2, it was observed that ammonium sulfate had a rather remarkable stimulating effect on the reaction as shown in figure 4. The effect of salts on the reaction is described in more detail in a later paper (1).

In subsequent work, the assay was simplified, and the sensitivity was increased, by omitting both buffer and cyanide, and adding 1 ml of 4 M $(NH_4)_2SO_4$. [Cyanide could be omitted because the salt inhibited the dark cytochrome oxidase.] Reduced cytochrome, Factor 2, and $(NH_4)_2SO_4$ were added to sufficient water to make a final volume of 3 ml, the mixture was allowed to come to 20° C, a suitable amount of Factor 1 was added in the dark, the optical density was measured, and the reaction was started by switching on the light, all as previously described (3).

STABILITY TO LIGHT, pH, AND HEAT: Exposure to a 300 watt reflector bulb at a distance of 10 inches for 70 minutes at 15° C had no effect on the activity of either Factor 1 or Factor 2. Material kept in total darkness for several hours had the same activity as the material exposed to light.

[4] C. Ardao and B. Vennesland, unpublished observations.

Figure 5 shows the effect of exposing Factor 1 and Factor 2 to different pH values at room temperature for 30 minutes. For Factor 1, there was a relatively broad range of stability from pH 5.0 to 11, with rapid inactivation below pH 4 and above pH 12. Factor 2 was decidedly more stable at pH 4, and 60 % of the activity was retained at a pH close to zero. This observation was repeated many times. In the presence of saturating concentrations of $(NH_4)_2SO_4$, however, Factor 2 was inactivated completely below pH 3.5.

Factor 1 lost no activity in 2 hours at 40° C but 90 % of the activity disappeared after storage for a week at room temperature. About half of the activity was lost in half an hour at 50° C, and inactivation was complete in 5 minutes at 70° C. These tests were carried out with the "solution" of Factor 1 in 1 % digitonin, prepared as previously described. No heat coagulum appeared, even on boiling. Factor 2 was somewhat more stable to heat than Factor 1. There was no loss of activity in 5 minutes at 60° C, but considerable inactivation generally occurred at 70° C, with different preparations giving different results at this temperature. Heat inactivation of Factor 2 was always complete in 10 minutes at 90° C.

Further Purification of Factor 2: On ultracentifugation, preparations of Factor 2 made in the standard fashion showed only 1 symmetrical peak which sedimented very slowly with an S_{20} of about

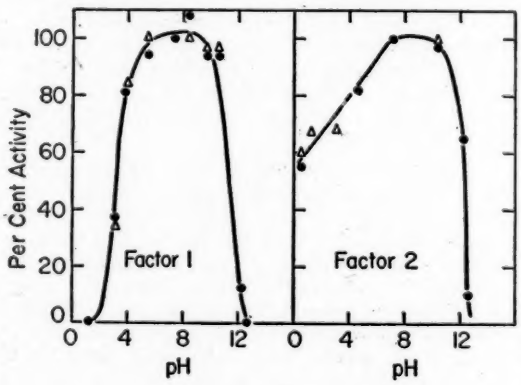

Fig. 5. Stability of Factor 1 and Factor 2 at different pH values. Triangles and circles represent experiments conducted with different preparations. The pH was adjusted by addition of HCl or NaOH to the desired point, allowed to remain there at room temperature for 30-minutes, then brought back to neutrality with NaOH or HCl. For controls, the same quantities of acid and alkali were mixed prior to addition to the sample. The assays were conducted with an amount of Factor 1 containing 41 μg of chlorophyll, and 0.1 ml of a preparation of Factor 2. In this system, both Factor 1 and Factor 2 were rate limiting. Half of the amount of Factor 1 gave 62 % of the activity. Half of the amount of Factor 2 gave 67% of the activity.

0.7 S. Electrophoresis of such preparations, however, showed their lack of homogeneity. In veronal buffer of pH 8, 3 peaks were readily distinguishable. Physical separation of the fractions indicated that the activity was associated with the middle peak. A 5-fold increase in the specific activity of Factor 2 was achieved by the following procedure. The starting material for this fractionation was the acetone precipitate of Factor 2, prepared as previously described.

The product obtained from 1 kg of spinach leaves was dissolved in 10 ml of 0.01 M potassium phosphate buffer of pH 7.0. The solution, containing 1.5 to 2.0 mg of protein per ml was diluted with 40 ml of water and 23.5 g of solid $(NH_4)_2SO_4$ were added slowly with constant stirring in the cold. The precipitate was removed by centrifugation. About one tenth of the activity was present in this fraction, but it was of low specific activity and was discarded. To the supernatant solution, 9 g of solid $(NH_4)_2SO_4$ were added as before, and the second precipitate was also recovered. Between one fourth and one third of the activity was present in this fraction, which had the same specific activity as the starting material. The supernatant, which contained most of the activity was close to saturation with $(NH_4)_2SO_4$ at 4° C. Acetic acid was added carefully, with rapid stirring in the cold, to bring the pH to 3.6. A precipitate began to form at pH 3.9, and acid was added until no more precipitate formed. Care was required to add no excess acid, since this could result in total destruction of the enzyme. The precipitate, which was almost white in color, was recovered by centrifugation after 2 hours standing in the cold. It contained about one third of the original activity and had a specific activity at least 3-fold that of the Factor 2 preparation. It was dissolved in 5 ml of 0.01 M phosphate buffer of pH 7.0 and dialyzed against the same buffer for 6 hours at 0° C. This preparation was designated Factor 2 A. No detectable Factor 2 activity remained in the supernatant solution from the isoelectric ammonium sulfate precipitation.

The final stage in fractionation was achieved by dropwise addition of 3.5 volumes of acetone (precooled to −15° C) to the solution of Factor 2 A. The precipitate, which was collected by centrifugation, contained one sixth to one fifth of the activity of the starting material, and had a specific activity at least twice that of Factor 2 A. This preparation was called Factor 2 B, and represented the highest purification achieved. The supernatant from the acetone precipitation still contained some enzyme, which could be recovered by further addition of a volume of acetone equal in amount to that used previously. The precipitate so obtained had about the same specific activity as Factor 2 A.

None of the purified preparations of Factor 2 were completely free from pigment. All samples had a small light absorption throughout the visible range. The absorbance increased very slowly with decreasing wave-length, sometimes giving a barely perceptible shoulder at 340 mμ, and always showing a peak or a

shoulder at about 270 mμ. The sedimentation pattern of Factor 2 was identical with that of Factor 2 A.

DISTRIBUTION OF FACTOR 2 IN SPINACH LEAF: Five sixths of all the Factor 2 activity in the chloroplasts could be obtained in solution by extraction of the chloroplasts with 0.05 M TRIS buffer of pH 8.0. This activity was precipitated with acetone in the usual manner, and attempts were made to conduct the purification with this preparation as starting material. Similar attempts were made to use the Factor 2 fraction prepared from cytoplasm as the starting material for further purification. These efforts did not indicate that there was any advantage in starting with these fractions rather than with Factor 2 prepared from digitonin extracts of chloroplasts, even though the cytoplasm contained more total activity than was obtained by digitonin treatment of the chloroplasts. Factor 2, as prepared by the standard procedure, was already purified to a considerable extent, as indicated by the data in table I, which shows the distribution of Factor 2 in various fractions in the spinach leaf, as well as typical results of a purification procedure.

For the determination of the activity present in the cytoplasm, leaves were ground in the NaCl-Versene suspending medium, as described for the preparation of washed chloroplasts. The chloroplasts were packed hard by 1 prolonged centrifugation and the supernatant was poured off and fractionated with acetone as described for digitonin extracts. The second acetone precipitate was dissolved in buffer and assayed. Because of interfering reactions, it was not possible to assay the cytoplasm directly, nor could the Factor 2 activity of chloroplasts be determined directly. The figure given for the total activity of chloroplasts was obtained by adding together the units found in the various fractions. The fractions listed under the heading "chloroplasts" represent various stages in the preparation of Factor 2. "Residue" is the green material remaining after 2 digitonin extractions. The Factor 2 content of Factor 1 was determined by working up a precipitate of Factor 1 by the procedure used to separate Factor 2 from chloroplasts. The "first acetone precipitate" represents material which was discarded in the preparation of Factor 2. Factor 2 is the standard preparation obtained as the second acetone precipitate, with which most of the experiments described in this paper have been carried out. Factor 2 A and 2 B represent successive stages in the purification of Factor 2, carried out as already described. The rates given in table I may be regarded as average, but individual preparations of Factor 1 and Factor 2 from different batches of spinach varied somewhat in activity, and it is clear that cross-standardization is required if different preparations are to be rigorously compared. The problem of standardization has not been completely solved. The total activity of the recombined fractions from separate preparations did not usually vary more than 2- to 3-fold when expressed in terms of the rate per mg of chlorophyll of Factor 1 with a given amount of protein of Factor 2.

TABLE I

DISTRIBUTION OF FACTOR 2 IN SPINACH LEAF, AND RESULTS OF PURIFICATION

PREPARATION	UNITS/G FRESH WEIGHT	SPECIFIC ACTIVITY ESTIMATED** ORIGINAL	SPECIFIC ACTIVITY ASSAYED
Cytoplasm	10.7	0.0005	0.02
Chloroplasts	6.6*	0.0006	
Residue	0.95		0.11
Factor 1	0.63		0.09
First acetone precipitate	1.55		0.09
Factor 2	3.50		0.21
Factor 2 A	1.15		0.58
Factor 2 B	0.52		1.00

One unit of Factor 2 is defined as that amount which causes an increase of 0.001 per minute in the rate of decrease of optical density, as measured in the standard ammonium sulfate assay system, with an amount of Factor 1 containing 25 μg of chlorophyll. The specific activity is defined as the number of activity units per μg of protein, determined according to Lowry et al (2).

* Calculated by addition.

** Estimated from the activity recovered and the total protein present. These values are only approximations.

SUMMARY

A procedure is described for fractionating cytochrome c photooxidase into 2 heat-labile components, one of which contains chlorophyll bound in a lipoprotein complex containing both phospholipid and nucleic acid. This component, called Factor 1, is insoluble in water, except in the presence of digitonin. The other component, called Factor 2, is water soluble and can be purified by fractional precipitation with acetone and ammonium sulfate. The best preparations of Factor 2 are about 1000 times more active on a protein basis than the whole leaf. The photooxidation of cytochrome c is shown to be dependent on the simultaneous presence of both of these components.

The authors are indebted to Drs. Hans Gaffron and Norman Bishop for stimulating discussion, and to Dr. E. Goldwasser for assistance and guidance in making the electrophoretic analyses and determining the sedimentation patterns in the ultracentrifuge.

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COMPARISON OF THREE PHOTOCHEMICAL ACTIVITIES OF CHLOROPLASTS[1,2]

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The Hill reaction is one of the simplest chloroplast reactions in which one can measure in unambiguous terms both the reducing and the oxidizing equivalents produced in the process of photosynthesis. In its simplest aspect, the oxidizing power is measured as oxygen production, while the reducing power is measured in terms of reduction of some electron acceptor (9). Two other photochemical reactions catalyzed by plant chloroplasts are the photochemical oxidation of ascorbic acid in the presence of the dye 2,6-dichlorophenolindophenol (DPIP), and the photochemical reduction of the dye indigo carmine (IC), which photoreduction requires the presence of both ascorbic acid and DPIP (10, 12). These reactions also can be described in terms of the classical Hill reaction, in which case one could consider the ascorbate-DPIP couple as reacting with the photochemical oxidizing system, allowing the photochemical reducing system to react with oxygen and indigo carmine respectively (10). Although there is to date no decisive experimental proof to show that such is the case, isotopic evidence does support this hypothesis (4). This paper describes the effect of various chloroplast treatments upon the ability of chloroplasts to catalyze a conventional Hill reaction, the photochemical oxidation of ascorbic acid in the presence of DPIP, and the photochemical reduction of indigo carmine, the purpose being to see if one can in this manner correlate these 3 photochemical activities of the chloroplast.

METHODS AND MATERIALS

The dye, 2,6-dichlorophenolindophenol, was obtained from Eastman Organic Chemical Co., Rochester, New York. Indigo carmine dye was obtained from National Aniline Division, Allied Chemical and Dye Co., New York, New York. Tris(hydroxymethyl) amino methane was obtained from Sigma Chemical Co., St. Louis, Mo. Digitonin was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. All other chemicals were obtained from commercially available sources and were of reagent grade. Water low in ion content was prepared by filtering distilled water through a column, approximately 4 cm in diameter and 28 cm long, filled with Amberlite MB-3 resin.

To prepare chloroplasts, 50 g of washed sugar beet leaves without petioles were blended for 50 seconds in a Waring blendor containing 100 ml of buffer solution (0.01 M KCl, 0.02 M phosphate, pH 7.0). After straining this homogenate through cheesecloth, the resulting fluid was centrifuged and the pellet coming down between 200 × G and 4000 × G (containing chloroplasts and chloroplast fragments) was washed once and resuspended in buffer solution.

The digitonin extract of the chloroplasts was prepared by incubating washed chloroplasts in 1 % digitonin solution for 15 minutes. Sonic oscillation of the chloroplasts was accomplished by a 10 minute treatment in a 10 K.C. Raytheon sonic oscillator. Ice water was used to keep the chamber cool. For incubation of the chloroplasts at pH 8.5 or pH 5.5, 0.01 M tris(hydroxymethyl) amino methane, pH 8.5, and 0.01 M acetate buffer, pH 5.5 were used. The treatment with water of low ion content consisted of an incubation for 30 minutes. Following these treatments the various suspensions were centrifuged for 10 minutes at 23,000 × G at 0° C. The supernatant solution from the digitonin-treated preparation was used as the digitonin extract. For the other preparations, the pellet was resuspended in buffer solution and used in the various tests.

Photoreduction of DPIP, used as a measure of the Hill reaction, was followed spectrophotometrically at 590 mμ. The reaction mixture consisted of 10 micromoles (μM) of phosphate buffer, pH 7.0, 0.5 μM of DPIP, chloroplast suspension equal to 0.1 mg of chlorophyll and water in a total volume of 3.0 ml.

The photoreduction of indigo carmine was determined essentially as described by Vernon and Hobbs (10), except that phosphate buffer at a pH of 7.0 was used. The reaction mixture contained 0.2 μM of indigo carmine dye, 1.0 μM of DPIP, 10 μM of ascorbic acid, 100 μM of phosphate buffer, pH 7.0, and from 0.1 to 0.2 mg of chlorophyll from the chloroplast preparations.

Photooxidation of the DPIP-ascorbate couple was determined using a modification of the method of Vernon and Ihnen (11), which consisted of titrating the remaining ascorbic acid with an excess of DPIP and using the spectrophotometer to determine the excess DPIP in the illuminated and dark reactions. Excess ascorbic acid was used in the reaction mixture since Vernon and Ihnen showed that the rate of the reaction was considerably greater when an excess was present. The reaction mixture consisted of 10 μM phosphate buffer, pH 7.0, 5 μM DPIP, 10 μM ascorbic acid, and 0.1 mg of chlorophyll from the chloroplast preparations in a final volume of 3.0 ml.

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Chlorophyll was determined by the method of Arnon (1). All spectrophotometric measurements were made with a Beckman Model DU spectrophotometer. The illumination time was 4 to 8 minutes and a light intensity of 4000 ft-c was employed. For each series of experiments a control of similar composition was used. Typical reaction rates for the control reactions are as follows: 1.5 μM IC photoreduced per mg chlorophyll in a 4 minute period, 1.9 μM DPIP reduced per mg chlorophyll in an 8 minute period for the Hill reaction, and 2.6 μM ascorbic acid photooxidized per mg chlorophyll for an 8 minute period.

Results

Of the various chloroplast treatments examined, 2 were found which resulted in extensive inhibition of the Hill reaction, while leaving the IC photoreduction and the photooxidation of ascorbic acid relatively unaffected. Figure 1 presents the results obtained after heating the chloroplast preparation at 51° C for 10 minutes and also following incubation at pH 8.5 for 30 minutes. The ability of chloroplasts to catalyze the Hill reaction was most sensitive to heating at 51° C, but both treatments showed preferential destruction of activity in the Hill reaction. The lability of the system responsible for the Hill reaction at high pH values agrees with data reported by Punnett (8). The possibility that this treatment extracted a factor from the chloroplasts which was necessary for complete activity was examined by adding to the reaction mixture an aliquot of the buffer in which the chloroplasts were incubated. No significant increase in activity was noted. Consequently, it would appear that the incubation at pH 8.5 inactivates some reaction component necessary for the Hill reaction to proceed, but not necessary for the other 2 photochemical reactions to proceed. The component destroyed by these treatments could be operative in the oxygen evolving system.

Four treatments were found which inhibited the IC photoreduction more severely than the other 2 photochemical activities. Sonic oscillation for 10 minutes was most effective in destroying indigo carmine photoreduction activity, as shown in figure 2. Treatment with sodium azide was less striking concerning inhibition of IC photoreduction. Exposure to ion low water and to a pH of 5.5 also resulted in approximately 50 % inhibition of IC photoreduction while the other 2 photochemical activities were only slightly affected, as shown in figure 3. The stability of the Hill reaction to a short treatment with water of low ion content was to be expected from data previously reported (2, 13). The lability of the system responsible for IC photoreduction to sonic oscillation was surprising, since this treatment did not appreciably affect the ability of the chloroplasts to catalyze the Hill reaction. Sonic oscillation for longer periods of time served to decrease the activity of the chloroplasts in the Hill reaction also, indicating that disruption of the chloroplast beyond a certain stage would prevent the Hill reaction from proceeding. It was not possible to show that any factor necessary for IC photoreduction was extracted by incubation in water of low ion content or at pH 5.5, so apparently such treatments inactivate some component specifically required for IC photoreduction, and again serves to illustrate that the 3 photochemical systems are not entirely similar.

The effect of $ZnSO_4$ upon the 3 chloroplast activities is shown by figure 4. This was the only case during this investigation that the photooxidation of ascorbic acid was reduced more than the other 2 photochemical activities. This inactivation may be due to displacement by the zinc ion of some other necessary metal ion in this system, or to the ability of zinc ion to complex some compound necessary for this reaction. The slight inhibition of the Hill reaction agrees with results reported by MacDowell, since he found that zinc sulfate prevents the action of many inhibitors (6) and it would not itself be expected to greatly inhibit this reaction.

Treatment of a chloroplast preparation with digitonin, followed by centrifugation, yielded a digitonin extract which was almost devoid of activity in the Hill reaction and IC photoreduction. The digitonin extract, however, was more than 6 times as active in the photooxidation of ascorbic acid on a chlorophyll basis, than the original chloroplasts as shown in figure 5. This can be compared with the report of Nieman and Vennesland (7), that digitonin extracts of chloroplasts are capable of catalyzing a photooxidation of cytochrome c in the presence of oxygen. In terms of enzymatic complexity it would be expected that both the photooxidation of ascorbic acid and the photooxidation of cytochrome c would be much more simple than the system necessary for both the Hill reaction and IC photoreduction. Apparently the digitonin ruptures the photosynthetic apparatus, leaving only a primative system capable of functioning in photooxidations.

In addition to the treatments listed above, there were several chloroplast treatments which resulted in about equal destruction of the ability of the chloroplasts to catalyze the Hill reaction and IC photoreduction, while leaving the photooxidation of ascorbic acid almost unaffected. Incubation with 8-hydroxyquinoline at 10^{-3} M final concentration resulted in a decrease of about 20 % in ability to carry out the Hill reaction and photoreduce IC. Digestion with lipase resulted in a 50 % decrease in these activities, while digestion with pancreatin caused a loss of about 70 % for these 2 activities. The treatments mentioned above did not appreciably affect the photooxidation of ascorbic acid. Incubation with phenyl mercuric acetate completely inhibited the activity in the Hill reaction and IC photoreduction, while affecting the photooxidation of ascorbic acid only to the extent of a 40 % inhibition. Digestion with lysozyme, or incubation with 2,4-dinitrophenol caused only a slight inhibition for all 3 activities.

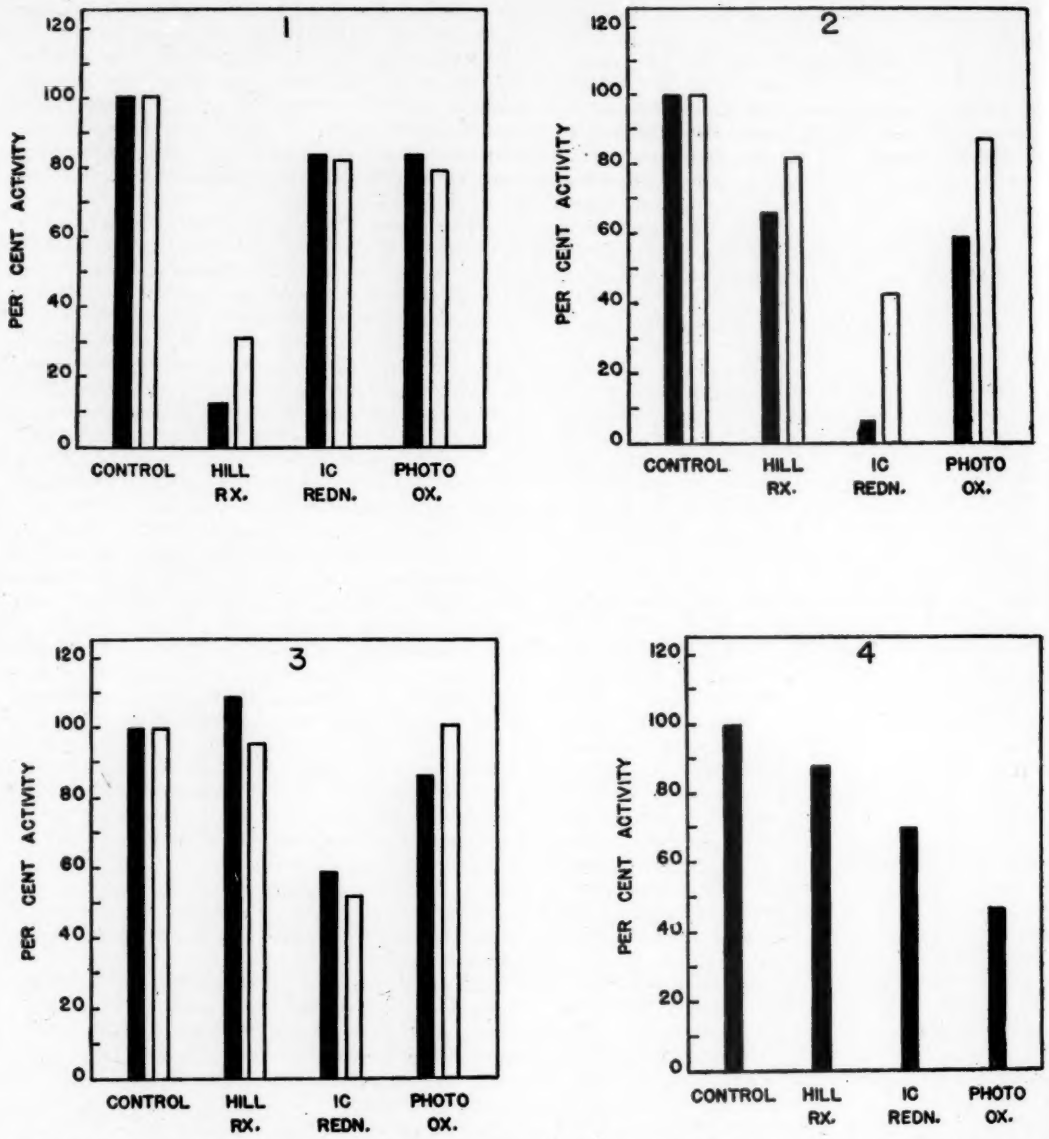

FIG. 1. Treatments preferentially inhibiting the Hill reaction. The solid bar represents data obtained upon heating chloroplasts for 10 minutes at 51° C. The open bar represents data obtained upon incubation of chloroplasts for 30 minutes at pH 8.5 as described in the section on Methods.

FIG. 2. Treatments preferentially inhibiting the photoreduction of indigo carmine. The solid bar represents data obtained after chloroplast exposure to sonic oscillation for 10 minutes. The open bar represents data obtained upon addition of sodium azide at a final concentration of 10^{-3} M to the reaction mixture.

FIG. 3. Treatments preferentially inhibiting the photoreduction of indigo carmine. The solid bar represents data obtained following incubation of chloroplasts in water of low ion content for 30 minutes. The open bar represents data obtained upon incubation of chloroplasts at pH 5.5 for 30 minutes as described in section on Methods.

FIG. 4. The effect of zinc ion upon the 3 photochemical activities. $ZnSO_4$ at a final concentration of 10^{-3} M was added to the standard reaction mixtures.

Discussion

The intent of the present investigation was to see if the 3 photochemical reactions followed were similarly affected by various treatments of the chloroplasts, in order that the degree of similarity of the 3 reactions could be assessed. From the data presented, it appears that they undoubtedly differ in some of the components involved in the reactions. In general, it appears that the photochemical oxidation of ascorbic acid in the presence of DPIP is the least sensitive of the 3 reactions, being less affected by all the treatments. Only addition of zinc ion resulted in less ability to photooxidize ascorbic acid, when compared to the other photochemical activities. This general insensitivity is further emphasized by the activity obtained in digitonin extracts of chloroplasts. Such preparations lost almost completely the ability to catalyze the Hill reaction and the photoreduction of IC, but their ability to catalyze the photooxidation of ascorbic acid was markedly increased. This would be in accord with the proposed mechanisms for the photochemical oxidation of ascorbic acid (12), in which the oxidative system produced in the primary photochemical reaction is thought to react with reduced DPIP, which in turn becomes reduced again by interaction with the ascorbate. In the proposed mechanism, the reducing power would be simultaneously causing the reduction of oxygen to the level of hydrogen peroxide. Thus, the enzyme systems involved in oxygen evolution would not be involved. Likewise, the interaction of oxygen with the photochemical reducing power would probably be through the mediation of riboflavin-5′-phosphate or some related compound, which would be relatively insensitive to the treatments used here. It would appear that the photooxidation of ascorbic acid in the presence of DPIP requires only the chlorophyll system responsible for the initial photochemical act of photosynthesis and a minimum of associated enzymes.

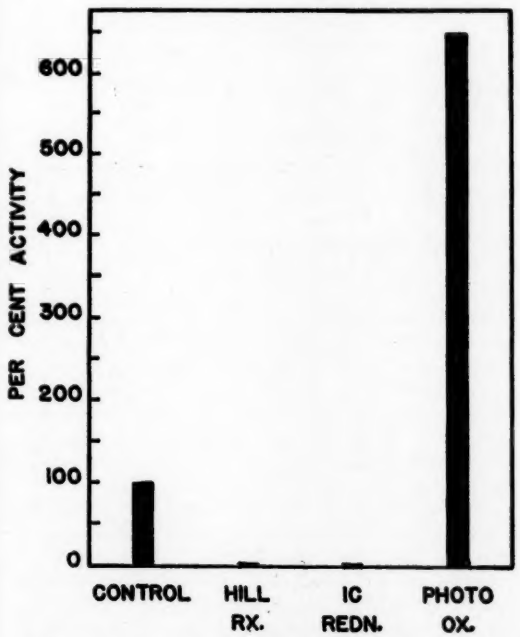

Fig. 5. Activity of digitonin extract in the 3 photochemical reactions. See section on Methods for details of preparation of digitonin extract.

The photoreduction of IC and the Hill reaction are both much more sensitive to the various treatments described than is the photooxidation of ascorbic acid. However, with the treatments described in this investigation, it is possible to preferentially inhibit either of these 2 reactions. In looking for an explanation of this behavior, one should consider that the salient differences between the Hill reaction and the IC photoreduction reaction are 1) the requirement for the presence of DPIP and ascorbic acid for IC photoreduction and 2) the difference in the oxidation potentials for the electron acceptors used in these reactions. The E'_0 for IC is -0.125 volts, while the E'_0 for DPIP is 0.217 volts at pH 7.0 (5).

The distinction between the Hill reaction and IC photoreduction, as shown by the treatments described in this investigation, could be due to interaction of IC and DPIP at different points in the photochemical reducing system. The potential difference in the 2 compounds would make this both possible and plausible. Another possibility for the differential effect of the chloroplasts treatments would concern the oxygen evolution system. If the DPIP and ascorbate are interacting with the photochemical oxidizing system as postulated (10), then those treatments which preferentially inhibit the Hill reaction could be destroying the enzyme system involved in oxygen evolution. From the experimental data available, it is not possible to say which of these possibilities is operative in each case. From the differential inhibition of the 2 reactions, one can say that there are different components involved in the 2 photochemical systems responsible for the Hill reaction and for IC photoreduction, but it is not possible to localize these different components.

Summary

1. The ability of chloroplasts to catalyze 3 photochemical activities following various treatments has been determined. The 3 photochemical activities determined were 1) the Hill reaction with 2,6-dichlorophenolindophenol (DPIP) as electron acceptor 2) the photoreduction of indigo carmine (IC) in the presence of ascorbate and DPIP and 3) the photochemical oxidation of ascorbate in the presence of oxygen and DPIP.

2. Heating chloroplasts at 51° C, and incubation at pH 8.5 for 30 minutes caused a preferential destruction of activity in the Hill reaction.

3. Sonic oscillation for 10 minutes, addition of sodium azide, incubation in ion low water, or incuba-

tion at pH 5.5 resulted in preferential destruction of IC photoreduction ability.

4. Addition of zinc ion preferentially inhibited the photooxidation of ascorbic acid.

5. Treatment of chloroplasts with digitonin resulted in a drastic loss of ability to catalyze the Hill reaction and IC photoreduction, yet yielded a preparation which was over 6 times as active in terms of ascorbic acid photooxidation.

6. These results are briefly discussed in terms of reaction mechanisms for these 3 photochemical reactions.

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UNCOUPLERS OF SPINACH CHLOROPLAST PHOTOSYNTHETIC PHOSPHORYLATION [1, 2, 3]

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It was recently observed by Arnon et al (3) that the formation of ATP[6] could occur during the course of the Hill reaction, with either ferricyanide or TPN as the Hill oxidant. The formation of ATP was stoichiometric with and dependent on the transfer of electrons to the Hill oxidant. The converse obtained to some extent also; that is, the Hill reaction with ferricyanide as oxidant proceeded approximately twice as fast if the phosphorylating reagents were present. We were able to repeat these observations of Arnon et al, including both aspects—ATP formation dependent on simultaneous electron flow, and electron flow stimulated by simultaneous phosphorylation (4). By using somewhat different conditions we found the stimulated rate of ferricyanide reduction to be from 3 to 3.5 times that of the control rate (7).

In investigating the mechanism by which this coupling occurs it may be useful to find reagents or physical treatments that lead to a disruption of the mechanism—i.e., uncouplers. A compound that is an uncoupler in this system, we will define as one that permits electron flow to proceed at a rapid rate in the absence of phosphorylation. Specifically it should meet the following criteria: a) in the phosphorylating Hill reaction, the reagent will inhibit the formation

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[6] Abbreviations to be used include: ATP for adenosine triphosphate, ADP for adenosine diphosphate, TPN for triphosphopyridine nucleotide, TRIS for tris (hydroxymethyl) aminomethane, DNP for dinitrophenol, PCP for pentachlorophenol.

of ATP but will not affect the rate of ferricyanide reduction and b) ferricyanide reduction in the absence of simultaneous phosphorylation will be stimulated by the reagent, at least as much as by phosphorylating reagents.

With these criteria now available, we have investigated two compounds that are effective as uncouplers of oxidative phosphorylation by mitochondria: dinitrophenol (11) and pentachlorophenol (15). Treatment of chloroplasts by dilution in sodium chloride at pH 6.0, which was previously found to activate them to a faster rate of ferricyanide reduction (12) is shown here to fulfill the criteria for uncoupling of the plastids. Arsenate was previously shown to replace phosphate in the stimulation of the Hill reaction (5); in the present work we show that arsenate fulfills one of the criteria for an uncoupling reagent. Finally we have found that ammonium ions are very effective uncouplers of chloroplast phosphorylation.

Materials and Methods

Chloroplasts were prepared from grocery spinach as described previously (6). Leaves were ground in 0.4 M sucrose, 0.01 M NaCl buffered at pH 7.8 with 0.05 M TRIS. The chloroplasts were washed once in the same medium. Chlorophyll was determined by the method of Arnon (1). Incorporation of P^{32} into ATP was determined by adsorbing the ATP^{32} on charcoal, washing the charcoal, then removing the labelled phosphate by hydrolysis (10).

Two kinds of reaction mixtures were employed in the present study. The first (control) contained 40 micromoles (μM) of TRIS buffer at pH 7.8, 70 μM of NaCl, 10 μM of $MgCl_2$, 15 μM of phosphate at pH 7.8, 2.0 μM of potassium ferricyanide, 0.1 μM of ATP and chloroplasts containing 0.030 mg of chlorophyll in a total volume of 3.0 ml. The optical density of the entire reaction mixture was determined at 400 mμ in a Beckman spectrophotometer; the cuvette was exposed to 5000 ft-c of white light from a tungsten lamp for 2 minutes, and then the decreased optical density was measured again. A 10 cm path of water was used as a heat shield, and control experiments had shown no changes with boiled chloroplasts.

The reaction mixture proper contained the same components as the control, and in addition 2.0 μM of ADP and approximately 2×10^5 cpm of P^{32}. Thus the only difference from the control mixture was the presence of ADP, which permitted net phosphorylation to occur. The rate of reduction in the complex control mixture is approximately 90 % of that seen in a much simpler control containing only chloroplasts, ferricyanide, buffer and NaCl (7), and the response to inhibitors was in all cases the same as in the simpler control. The complex control mixture was used in order to approximate as closely as possible the conditions in the phosphorylating reaction, except for the absence of net ATP formation.

After the amount of ferricyanide reduction had been measured, the reaction mixtures in the phosphorylation cuvettes were denatured by adding 0.3 ml of 20 % trichloroacetic acid, the mixture was centrifuged, and an aliquot was removed for ATP^{32} determination. In all experiments a zero time control was included in which all reagents were added directly to trichloroacetic acid; this control value for ATP^{32} was subtracted from all of the experimental measurements. The values shown in the tables represent the average of duplicate or triplicate determinations of both the rate of ferricyanide reduction and of phosphorylation. Duplicate values generally agreed within 10 %. Each experiment shown was repeated at least 4 times.

From the measurements obtained in the phosphorylating cuvettes it is possible to determine a P/2e ratio. This is defined here as micromoles of ATP formed per $1/2 \times$ no. of μ equivalents of Hill oxidant reduced, and is listed in the tables as the observed P/2e ratio. If the amount of ferricyanide reduced in the control cuvette is subtracted from that reduced in the phosphorylation cuvette, one determines the extra ferricyanide reduction due to simultaneous phosphorylation. Using this net value for electron flow a calculated P/2e ratio is obtained, defined as micromoles of ATP formed per $1/2 \times$ net μ equivalents of Hill oxidant reduced in the phosphorylating reaction. These calculated values are also listed in the tables.

With a variable source of leaves, the extent of stimulation of the Hill reaction due to simultaneous phosphorylation has varied between 2 and 3.5-fold. The experiments chosen for the tables were ones in which the degree of stimulation was in the upper range, since presumably these would be the better chloroplast preparations. In these experiments where the net stimulation of electron flow is relatively high, the calculated P/2e ratio is minimal.

The values shown in the tables are μ equivalents of ferricyanide reduced or micromoles of ATP formed in the 3 ml of reaction mixture. Multiplying any of these numbers by 1000 will give the μ equivalents or micromoles formed per mg chlorophyll per hour.

Results

Table I shows the effect of dinitrophenol on ferricyanide reduction in the presence or absence of simultaneous phosphorylation, and on the amount of ATP formed in the phosphorylation. It can be seen that dinitrophenol at 3.3×10^{-4} M, and even more at 1×10^{-3} M, inhibits ATP formation. These same concentrations also inhibit ferricyanide reduction either in the presence or absence of phosphorylation. The extent of the inhibitions of electron flow and of ATP formation are approximately equal in the phosphorylating Hill reaction.

Also shown in this table is the observed P/2e ratio which is close to 1.0. Note that in this experiment the net stimulation is 2.5 times as large as the control rate, and the total stimulated rate is 3.5 times as great as the control. The calculated P/2e ratios range

from 1.3 to 1.7 in this experiment, and in general they vary from 1.3 to 2.0 in our experience.

Results with pentachlorophenol are shown in table II. As with dinitrophenol, both ATP formation and ferricyanide reduction are inhibited in the phosphorylation cuvette and at the same concentrations of pentachlorophenol. The control rate, however, is hardly affected except for a slight stimulation at 1×10^{-5} M.

Although the observed P/2e ratios drop from 0.84 to 0.42 when phosphorylation still occurs, the calculated P/2e ratio remains between 1.25 and 1.55. The difference is due to the failure of pentachlorophenol to inhibit the control rate.

Since the basal rate is stimulated 20% by 1×10^{-5} M pentachlorophenol, it was thought that the reagent might have some uncoupling ability which could not show up due to the brief (2 minute) exposure time. However, even after a 60 minute preincubation of chloroplasts with 1×10^{-4} M pentachlorophenol no further stimulation of the basal rate was seen (final concentration in the reaction mixture after preincubation was 1×10^{-5} M). If there is any uncoupling effect it is only a minor one, and it is completely masked by the inhibiting action.

Chloroplasts were diluted, to a final concentration of 0.001 mg chlorophyll per ml, in 0.35 M NaCl at pH 6.0 and in agreement with previous results (12) their ability to reduce ferricyanide in the absence of phosphorylation more than tripled (table III). However, the rate of ferricyanide reduction in the untreated chloroplasts was stimulated to about the same level by phosphorylation, and the treated chloroplasts show almost no response to phosphorylating reagents. As might be expected, therefore, these treated chloroplasts make almost no ATP when provided with the phosphorylating reagents. The P/2e ratio, either observed or calculated, falls to a negligible value.

Arsenate (table IV) inhibits ATP formation by virtue of competition with phosphate (5). However ferricyanide reduction is quite unaffected, even when ATP formation is inhibited 65%. The P/2e ratios, both observed and calculated, drop steadily. The control rate is not stimulated by arsenate because ADP is absent. The stimulation by arsenate requires the presence of ADP (5).

It was observed accidentally that ammonium sulfate could induce a large increase in the rate of ferricyanide reduction by fresh chloroplasts, entirely in the absence of phosphorylating reagents. This is shown in table V, as is a 95% inhibition of ATP formation by NH_3 without any inhibition of ferricyanide reduction in the phosphorylating reaction. Actually

TABLE I

EFFECT OF DINITROPHENOL ON HILL REACTION PHOSPHORYLATION

DNP Concentration, M	Ferricyanide reduced −ADP	Ferricyanide reduced +ADP	ATP formed	P/2e Observed	P/2e Calculated*
0	0.24*	0.83*	0.39	0.94	1.4
1×10^{-4}	0.24	0.74	0.32	0.86	1.3
3.3×10^{-4}	0.19	0.45	0.22	0.98	1.7
1×10^{-3}	0.07	0.10	0.0	...	...

* Micromoles in 2 minutes. Reaction conditions described in Materials and Methods section.

TABLE II

EFFECT OF PENTACHLOROPHENOL ON HILL REACTION PHOSPHORYLATION

Pentachlorophenol concentration M	Ferricyanide reduced −ADP	Ferricyanide reduced +ADP	ATP formed	P/2e Observed	P/2e Calculated
0	0.26	0.76	0.32	0.84	1.3
1×10^{-6}	0.25	0.75	0.31	0.83	1.2
3.3×10^{-6}	0.29	0.51	0.17	0.67	1.6
1×10^{-5}	0.31	0.43	0.09	0.42	1.5
3.3×10^{-5}	0.25	0.27	0.0	...	...

TABLE III

HILL REACTION PHOSPHORYLATION BY CHLOROPLASTS DILUTED IN NaCl AT pH 6.0

Chloroplasts	Ferricyanide reduced −ADP	Ferricyanide reduced +ADP	ATP formed	P/2e Observed	P/2e Calculated
Fresh	0.17	0.62	0.32	1.03	1.4
Diluted	0.49	0.53	0.004	...	...

the reduction of ferricyanide is slightly stimulated as ATP formation disappears.

These effects are shared by ammonium chloride, ammonium formate and ammonium acetate, and so are due to the cation and not the anion. Sodium, potassium, magnesium or calcium ions do not replace the ammonium ions. The effect of a given concentration of ammonium salt is not increased at all by preincubation of the chloroplasts with the salt. The concentration of ammonium ions needed for 50 % inhibition of ATP formation varies between 6×10^{-4} and $4 \times^{-3}$ M in our experience.

An unusual aspect of uncoupling by ammonium ions is that it seems to be freely reversible once the ammonium salt is removed by washing (table VI). Control chloroplasts either before or after a wash have a low rate of ferricyanide reduction, which is stimulated by the addition of phosphorylating reagents. Chloroplasts stored in 5×10^{-2} M ammonium chloride carry with them into the reaction mixture enough ammonium chloride to make the final concentration 1.67 $\times 10^{-3}$ M. This is enough so that they are about 65 % uncoupled in this experiment: the rate of reduction is high, and it is stimulated only slightly by phosphorylating reagents. Also this preparation makes only 31 % as much ATP as the control chloroplasts when provided with the appropriate reagents. These same chloroplasts, after 1 centrifugation and resuspension in the absence of ammonium chloride show an almost unimpaired coupled phosphorylation. The control rate of ferricyanide is back to a low value, and is stimulated by phosphorylating reagents. ATP formation is 85 % of that in untreated chloroplasts.

There is an extra complication to the effect of ammonium salts on chloroplasts, in that an excess is inhibitory to ferricyanide reduction. The inhibition is observed at alkaline pH only; while severe at 7.8 it is apparently negligible at 7.2. The sensitivity to inhibition by excess ammonia has varied with different chloroplast preparations; between 6×10^{-3} M and 2×10^{-2} M has been required for inhibition to appear.

TABLE IV

EFFECT OF ARSENATE ON HILL REACTION PHOSPHORYLATION

ARSENATE CONCENTRATION M	FERRICYANIDE REDUCED −ADP	FERRICYANIDE REDUCED +ADP	ATP FORMED	P/2e OBSERVED	P/2e CALCULATED
0	0.19	0.42	0.21	1.00	1.8
1.0×10^{-2}	0.20	0.44	0.19	0.86	1.6
1.5×10^{-2}	0.22	0.46	0.14	0.61	1.2
3.3×10^{-2}	0.22	0.48	0.07	0.29	0.5

TABLE V

EFFECT OF AMMONIUM SULFATE ON HILL REACTION PHOSPHORYLATION

NH_4 ION CONCENTRATION* M	FERRICYANIDE REDUCED −ADP	FERRICYANIDE REDUCED +ADP	ATP FORMED	P/2e OBSERVED	P/2e CALCULATED
0	0.21	0.59	0.36	1.22	1.9
2×10^{-4}	0.30	0.62	0.32	1.03	2.0
6.6×10^{-4}	0.62	0.74	0.11	0.30	1.8
2×10^{-3}	0.67	0.71	0.016	0.05	[0.8]
4.7×10^{-3}	0.53	0.53	0.00	...	...

* Concentration of the ammonium ion is shown; concentrations of ammonium sulfate were half those shown.

TABLE VI

REVERSIBILITY OF UNCOUPLING BY AMMONIUM CHLORIDE

CHLOROPLASTS	NH_4Cl* CONCENTRATION M	FERRICYANIDE REDUCED −ADP	FERRICYANIDE REDUCED +ADP	ATP FORMED	P/2e OBSERVED	P/2e CALCULATED
Control	0	0.19	0.62	0.34	1.10	1.6
Control after wash	0	0.21	0.65	0.32	0.99	1.5
Stored in NH_4Cl	1.67×10^{-3}	0.73	0.78	0.10	0.27	(4.0)
Stored in NH_4Cl after wash	0	0.23	0.47	0.27	1.15	(2.3)

* Chloroplasts were stored in 5×10^{-2} M ammonium chloride; on transfer to the reaction mixture they carried enough with them to make the final concentration equal to that shown.

Discussion

Prior to the discovery by Arnon et al (3) of ATP formation coupled to ferricyanide reduction, it was not possible to measure the rate of electron flow while ATP was being formed by chloroplasts. Consequently it was impossible to determine whether any particular inhibitor of phosphorylation was actually an uncoupler, or whether it was inhibiting electron transport or photolysis primarily and phosphorylation secondarily. Thus dinitrophenol was found to inhibit phosphorylation (2) and by analogy with its effect in mitochondria, would be presumed to be an uncoupler of chloroplast phosphorylation. However with the critical analysis possible now that phosphorylation and electron flow can be measured in the same reaction, we see quite clearly that dinitrophenol is an inhibitor of both reactions and so cannot be called an uncoupler. Similarly pentachlorophenol, which works as an uncoupler of oxidative phosphorylation at concentrations below those effective for dinitrophenol (15) inhibits both ferricyanide reduction and ATP formation by chloroplasts, and is therefore an inhibitor but not an uncoupler.

It is widely considered (8, 11) that the effects of dinitrophenol on oxidative phosphorylation are due to interference with the basic mechanism for the formation of ATP. Since DNP is not an uncoupler of photosynthetic phosphorylation, this may be an indication that the mechanism of ATP formation is different in chloroplasts than in mitochondria. Alternatively of course the mechanism might be very similar, but the specificity for uncouplers might be different due to dissimilar enzymes involved. Entirely different evidence has been presented elsewhere (5) which indicates that the mechanism for ATP formation in mitochondria is different from that in chloroplasts.

Arsenate was previously shown (5) to stimulate ferricyanide reduction providing ADP and magnesium were present. Arsenate is an uncoupler in mitochondria (10) but there is only a partial requirement for ADP in uncoupling oxidative phosphorylation. We see here that arsenate meets one of the criteria for an uncoupler, in that electron flow remains high even when ATP formation is inhibited seriously. The basal rate is not stimulated, however, due to the absence of ADP.

The procedure of diluting chloroplasts in 0.35 M NaCl at pH 6.0 was first developed as a method to permit them to reduce ferricyanide more rapidly (12). It was observed that this treatment also made them unable to phosphorylate in a cyclic system. However the complete proof that the dilution treatment results in uncoupling had to wait for the present experimental procedure, in which phosphorylation and electron flow are measured simultaneously.

The discovery that ammonium salts are uncouplers of the Hill reaction phosphorylation at 10^{-3} M or below was quite unexpected. Nevertheless it is clear that they meet both criteria for uncoupling quite well. The mechanism for this effect is still entirely unknown. However it is possible to rule out any irreversible alteration in chloroplast structure, since the uncoupling action is reversible by washing out the ammonium salt. Whatever ammonium does, it must be present continuously to exert its effect. The action of ammonium ions on photosynthetic phosphorylation might be a partial explanation for some of the well known toxic effects of ammonia in plant tissues (9, 14). Inhibition of cyclic phosphorylation by ammonium ions was observed previously by Ohmura (13).

There are at least two and probably more than two basic ways in which uncoupling can occur (see (7)). The experimental procedures we have used here to determine the existence of uncoupling are not adequate to determine what the mechanism is for a given reagent or treatment.

The observed P/2e ratios under the present conditions vary between 0.8 and 1.2, in the phosphorylating reactions with no inhibitory agent added. These ratios agree with those observed by Arnon et al (3). However, a basal rate of ferricyanide reduction always occurs in the entire absence of phosphorylation. It seems entirely conceivable that this amount of electron flow might continue to occur, entirely unassociated with the production of ATP, even when the additional electron flow does result in ATP formation as ADP, phosphate and magnesium are provided. If that is true then the only electron flow actually concerned with making ATP is that which comprises the net stimulation of the Hill reaction. The efficiency of the reactions actually concerned would then be better expressed by the calculated P/2e ratio than by the observed P/2e ratio. We have shown this calculated ratio in all of the experiments listed, and it varies from 1.3 to 2.0 or higher. While no great accuracy can be ascribed to the resulting numbers, they do indicate the possibility of a P/2e ratio greater than 1, and presumably approaching 2 as a limit.

If this calculation is valid, the resulting P/2e ratios suggest that there are 2 phosphorylating steps on the way from the first reduced product of photolysis to ferricyanide. The evidence that we have is very definitely inadequate to decide on the validity of the assumptions, however. A decision would be possible if chloroplasts were found in which electron transport was completely coupled to phosphorylation, so that subtraction of the control rate would be a negligible factor.

Summary

The criteria are indicated which can distinguish whether a compound uncouples electron transport from phosphorylation in the phosphorylating Hill reaction, or whether it is an inhibitor of both electron flow and ATP formation.

By these criteria, dinitrophenol and pentachlorophenol do not act as uncouplers in chloroplasts, but only as inhibitors. Dilution of chloroplasts in sodium chloride solution at pH 6.0 results in uncoupling. Arsenate acts as an uncoupler, but only in the presence of ADP and magnesium. Ammonium ions are very

effective uncouplers of photosynthetic phosphorylation. The action of ammonium salts are reversible by simply washing the chloroplasts.

It is suggested tentatively that a P/2e ratio of more than 1.0 might be possible for ATP formation coupled to ferricyanide reduction.

This work was greatly expedited by the assistance of Mrs. M. Evans.

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COMPARISON OF FERRICYANIDE AND 2,3',6-TRICHLOROPHENOL INDOPHENOL AS HILL REACTION OXIDANTS [1, 2, 3]

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With the development of a spectrophotometric assay of the Hill reaction with ferricyanide (9), a comparison between the rates of reduction of ferricyanide and the dye, 2,3',6-trichlorophenol indophenol by intact chloroplast appeared desirable. Previous attempts at such a comparison were hampered by the different methods used for the two oxidants. These differences are minimized by following the reduction of both oxidants spectrophotometrically. Under comparable circumstances, one observes that intact chloroplasts reduce ferricyanide at a much lower rate than that at which they reduce indophenol dye. This difference has been studied and a procedure has been devised which permits chloroplasts to reduce both acceptors at the same rate.

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[2] Contribution no. 258 from the McCollum-Pratt Institute.

[3] This work was supported in part by Grant no. RG 3923 from the National Institutes of Health, Public Health Service, and by Grant NSF 1298 from the National Science Foundation.

[4] Predoctoral Fellow of the National Cancer Institute, Public Health Service. This work was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology at Johns Hopkins University, Baltimore, Maryland. Present address: Biochemistry Department, University of Chicago, Chicago, Illinois.

Materials and Methods

Reagents: 8-Hydroxyquinoline and *a,a'*-dipyridyl were purchased from the Baker Chemical Company and Fisher Scientific Company, respectively. Triphosphopyridine nucleotide (TPN) was obtained from the Pabst Laboratories. 2,3',6-Trichlorophenol indophenol was a product of Eastmen Organic Chemicals.

Preparation of Chloroplasts: Fresh spinach was obtained at the local market, and whole chloroplasts were prepared from it either by the method of Jagendorf (7) or of Arnon et al (2). When sonicated chloroplasts were used, sonication was performed in

a 10-kilocycle Raytheon sonic oscillator. Freshly prepared chloroplasts were used in all experiments and Hill reaction activity was measured immediately after preparation of the chloroplasts, unless otherwise indicated.

MEASUREMENTS. All spectrophotometric measurements were performed with a Beckman Model B or DU spectrophotometer using 3.0 ml cuvettes having a light path of 1.0 cm. Hill reaction activity measurements with 2,3′,6-trichlorophenol indophenol were performed as described by Jagendorf (7). Measurement of ferricyanide reduction was accomplished as described by Krogmann and Jagendorf (9). As previously reported, the oxidant concentrations chosen for these measurements were 0.08 micromoles (μM) of the dye and 1 μM of ferricyanide per 3.0 ml final volume. Chlorophyll concentrations ranged from 0.003 to 0.016 mg per ml in the final reaction mixture. All Hill reaction activity measurements were made at room temperature (usually 25° C) with appropriate precautions to avoid a temperature rise during illumination. The light intensity was approximately 5000 ft-c in these experiments, unless otherwise noted. The light source was a 150-watt incandescent lamp. Control measurements had shown that dye reduction was dependent on the presence of active chloroplasts, and no direct photoreduction of the dye occurred under these conditions. The reaction mixture was buffered at p.H 7.2 with either 0.05 M TRIS or 0.05 M phosphate buffer, and included 0.01 M NaCl. Photosynthetic phosphorylation was measured according to the Arnon procedure (2) as modified by Jagendorf and Avron (8) with phenazine methosulfate used as the catalytic cofactor. Chloroplast reduction of TPN was measured as described by San Pietro (13). Chlorophyll concentration was determined by the method of Arnon (1).

RESULTS

HILL ACCEPTOR DIFFERENCES USING INTACT CHLOROPLASTS: When rates of ferricyanide and dye reduction in the Hill reaction were compared using thoroughly sonicated chloroplasts, very little difference was observed (9). However, when intact chloroplasts, prepared in 0.05 M phosphate buffer pH 7, 0.4 M sucrose, 0.01 M KCl medium were used, the rate of Hill reaction with ferricyanide was only 30 to 40 % of the rate with dye. An experiment was performed to check the degree of sonication necessary to equalize the rates to the 2 acceptors. Chloroplasts were diluted in the phosphate, sucrose, KCl medium and sonicated for varying intervals of time. The data obtained are presented in figure 1. The initially high dye reduction activity decreased steadily with increasing time of sonication. The initially low rate of ferricyanide reduction first increased with sonication, then decreased. After 120 seconds sonication, the rates to the 2 acceptors were almost equal.

EFFECT OF HYPOTONIC MEDIUM AND OF AGING: It was reasoned that a medium of very low tonicity

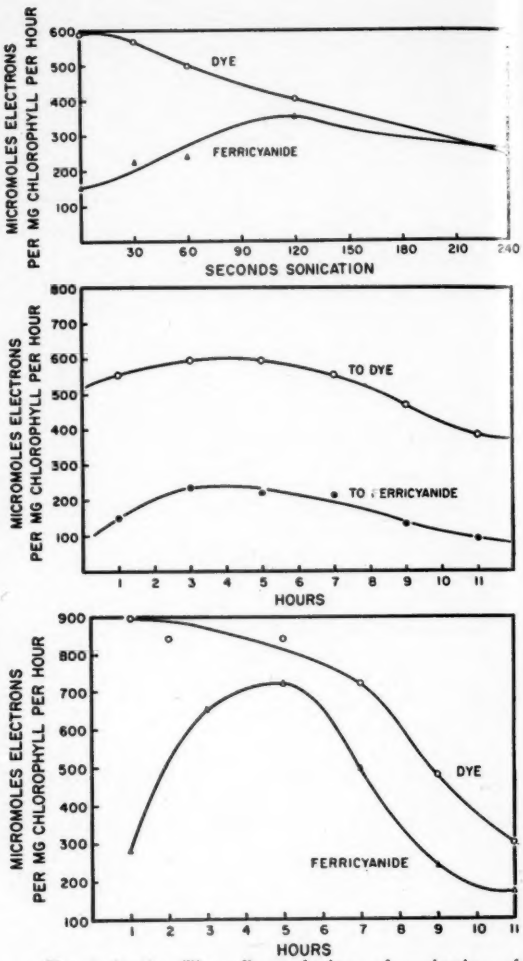


FIG. 1 (*top*). The effect of time of sonication of chloroplasts on the rates of 2,3′,6-trichlorophenol-indophenol and ferricyanide reduction by the Hill reaction. The chloroplasts were prepared and washed once in the phosphate-sucrose-KCl medium. The chlorophyll concentration during sonication was 0.01 mg per ml. Assays were performed as previously described, using sufficient chloroplast material to give a change in optical density of at least 0.2 after 30 seconds of illumination with saturating light.

FIG. 2 (*center*). The effect of aging chloroplasts in hypotonic medium on the rates of reduction of 2,3′,6-trichlorophenol indophenol and ferricyanide. The chloroplasts used here were prepared and washed 5 times in 0.35 M NaCl. The curves show the effect of aging chloroplasts in 10^{-3} M phosphate buffer pH 7 at zero degrees on the rate of Hill activity. The chlorophyll concentration of preparations during aging was 0.147 mg per ml.

FIG. 3 (*bottom*). The effect of aging chloroplasts in 0.35 M NaCl on the rate of Hill activity. Aging in NaCl was carried out at a chlorophyll concentration of 1.17 mg per ml at zero degrees.

might also equalize the rates to the 2 acceptors by causing disruption of the chloroplast structure. Chloroplasts prepared and washed 5 times in 0.35 M NaCl were used in this experiment. The final chloroplast suspension was divided in half, one half centrifuged, and the sedimented chloroplasts resuspended in 10 times the original volume of 10^{-3} M phosphate buffer pH 7. The other half was held as a control. Changes in Hill activity to the 2 acceptors were followed with time. As can be seen in figure 2, decreasing the tonicity of the medium had no immediate effect on ferricyanide reduction. Even prolonged exposure to hypotonic medium did not duplicate the effect of sonication. However, the control samples from the original 0.35 M NaCl suspension showed a 3-fold increase in ferricyanide reducing activity after 5 hours aging at zero degrees (fig 3).

Effect of Dilution: It soon became apparent that dilution of chloroplasts in NaCl, to a final chlorophyll concentration of approximately 0.01 mg per ml resulted in an immediate increase in the rate at which they could reduce ferricyanide in the light, even when the chloroplasts had been prepared from leaves ground in NaCl. The new rate of ferricyanide reduction approximately equalled the rate of dye reduction. The rate of dye reduction was hardly affected by this dilution process. Maximal values for reduction of the 2 Hill acceptors attained with the spectrophotometric assay of activated chloroplasts are presented in table I.

Effect of Hydrogen Ion Concentration: If the pH is maintained at neutrality by buffering the NaCl solution used for diluting the chloroplasts, ferricyanide reducing activity remains low (table II). The optimal pH for the increase in ferricyanide reducing ability turned out to be between 6 and 6.3 (fig 4). This pH refers only to the dilution step; the chloroplasts had been prepared in unbuffered NaCl and the assay was performed at pH 7. It should be noted that in the assay procedure the chloroplasts are diluted at least as much as they are in the dilution step.

Table I

Maximal Rates of Hill Activity

Preparation	Rates as μM of electrons/mg chlorophyll/hour	
	to dye	to ferricyanide
Activated chloroplasts avg of 6 preparations	912	803
Range	720 to 992	640 to 1219
Intact chloroplasts avg of 8 preparations	744	215
Range	583 to 936	120 to 273

Assays were performed as previously described using 10 to 50 μg chlorophyll per assay with a 30-second exposure to saturating light. In all cases, activation of the chloroplasts was achieved by dilution of the preparation in 0.35 M NaCl buffered at pH 6.0 to a final chlorophyll concentration of approximately 0.01 mg per ml.

The neutral pH during the assay presumably prevents activation during the course of the reaction.

The activation of chloroplasts to a high rate of ferricyanide reduction is apparently irreversible. As all assays were performed at pH 7, the activation by exposure to lower pH did not depend on the maintenance of that pH. When chloroplasts were centrifuged from a dilution medium buffered at pH 6.3 and resuspended in a minimal volume of low salt medium buffered at pH 7, the Hill reaction activity to ferricyanide remained high.

Effect of Salt and Chloroplast Concentration: The sodium chloride concentration of the diluent was varied in a series of experiments. Sodium chloride concentrations between 0.25 and 0.4 molar proved optimal. Lower concentrations failed to cause activation while exposure of chloroplasts to sodium chloride concentrations higher than 0.4 molar inhibited Hill activity. Inhibition of chloroplast fragments by sodium chloride was observed by Spikes et al who reported 50 % at 0.49 M (14).

Optimal activation required dilution of the chloroplasts to a final chlorophyll concentration of 0.01 mg per ml or less. This aspect was difficult to study quantitatively since activation would occur in the more concentrated suspensions, only at a much slower rate.

Effect of Light Intensity: The rate of the Hill reaction was determined as a function of light intensity between 25 and 2000 ft-c, before and after chloroplast activation. The resultant curves are plotted in figure 5, in the form of 1/rate vs 1/light intensity, as suggested by the kinetic treatment of Lumry, Spikes and Eyring (12). In this formulation the slope of the line is related to 1/rate constant for the limiting light reaction, and the intercept is related to

Table II

Maximal Rate of Hill Activity Attained under Various Conditions of Chloroplast Dilution

Dilution treatment	Chlorophyll concentration before assay mg/ml	Rate as μM of electrons/mg chlorophyll/hour	
		to dye	to ferricyanide
Control	1.2	600	220
Diluted 100 : 1 with 0.35 M NaCl	0.012	720	640
Diluted 100 : 1 with 0.35 M NaCl, 0.01 M phosphate buffer, pH 7.	0.012	630	255
Diluted 100 : 1 with 0.35 M NaCl 0.01 M TRIS pH 7.	0.012	684	276

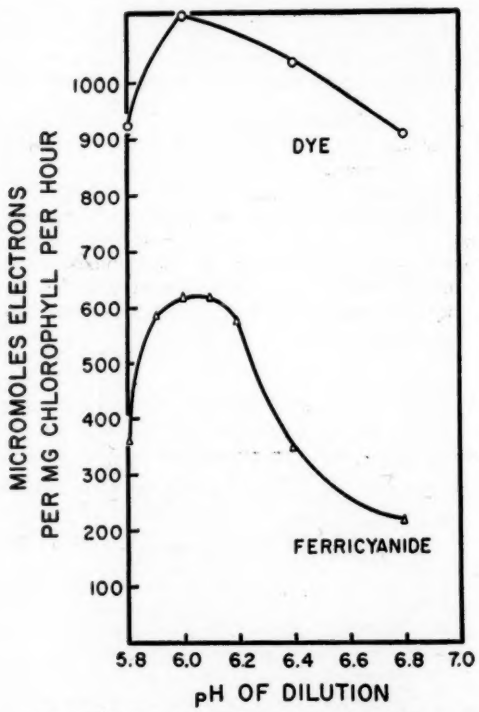
The chloroplasts used here were prepared and washed once in 0.35 M NaCl prior to dilution. The assays were performed as previously described, using 10 to 50 μg chlorophyll per 3 ml reaction mixture with a 30-second exposure to saturating light.

1/rate constant for the limiting dark reaction. With this analysis it is evident that the dilution treatment has caused a large increase in the limiting dark reaction, but has increased the limiting light reaction only about 50 %.

INHIBITION BY METAL CHELATORS: Activated and control chloroplasts were compared with regard to light dependent ferricyanide reduction (table III). The preparation diluted at pH 6.2 is seen to reduce ferricyanide 3 times as fast as does the control preparation. The 2 sets of chloroplasts were practically identical in their ability to reduce trichlorophenol indophenol. They were then compared in regard to sensitivity to 8-hydroxyquinoline and a,a'-dipyridyl, using indophenol dye as the oxidant. The Hill reaction activity of the treated chloroplasts is much more sensitive to inhibition by chelating agents than is that of the control chloroplasts. Thus the treatment which increases the activity of the chloroplasts in photoreduction of ferricyanide also increases the sensitivity of the chloroplasts to these 2 inhibitors.

CORRELATION WITH PHOTOSYNTHETIC PHOSPHORYLATION: An attempt was made to correlate the levels of ferricyanide reducing ability with the process of photosynthetic phosphorylation. Cyclic phosphorylation was studied, using the experimental procedure of Arnon et al (2) as modified by Jagendorf and Avron (8). Phenazine methosulfate was the catalytic cofactor employed. The data from triplicate experiments are presented in table IV. It can be seen that chloroplasts which show the greatest Hill activity with ferricyanide have the least ability to carry out phosphorylation.

CORRELATION WITH PYRIDINE NUCLEOTIDE REDUCTION: Next a correlation was sought between the ability to photoreduce ferricyanide and pyridine nucleotide. In these experiments, the chloroplasts were saturated with the pyridine nucleotide reducing enzyme from spinach described by San Pietro (13). Chloroplasts were made limiting in the reaction mixture to reveal differences in their abilities to participate in the reduction of TPN. As can be seen in table V,



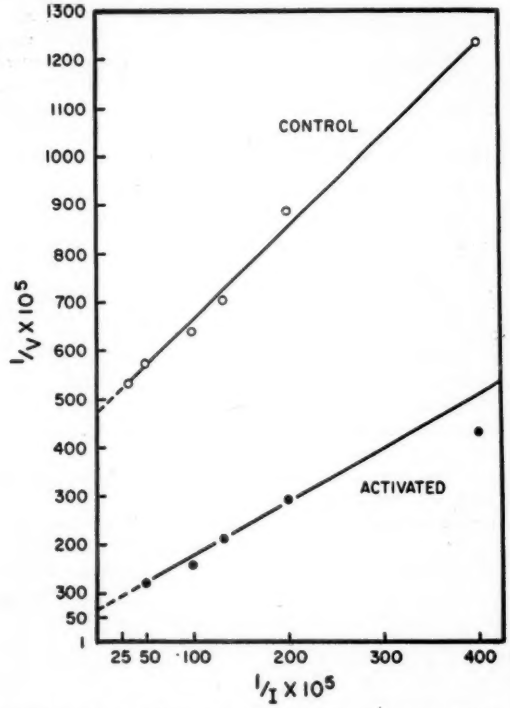


FIG. 4 (*left*). The effect of pH of dilution pretreatment on the rate of reduction of 2,3',6-trichlorophenol indophenol and ferricyanide by the Hill reaction of whole chloroplasts. The chlorophyll concentration of the diluted chloroplast suspension equals approximately 0.01 mg per ml. Assays were performed as previously described using 10 to 30 μg chlorophyll per 3 ml reaction mixture with a 30-second exposure to saturating light.

FIG. 5 (*right*). The effect of light intensity on the rate of ferricyanide reduction by chloroplasts before and after activating pretreatment. Assays were performed as previously described. The control chloroplasts were assayed at 30 μg chlorophyll per 3 ml reaction mixture and the activated chloroplasts at 7.5 μg. The upper line (O) is that obtained with control chloroplasts. The lower line (●) was obtained with the same chloroplast preparation diluted 100-fold in 0.35 M NaCl buffered at pH 6. Light intensities were varied by placing the cuvettes at various distances away from the tungsten lamp; the intensity was measured at each distance by a Weston light meter. The units of light intensity are ft-c, those for velocity are μ equivalents of ferricyanide reduced.

TABLE III

CORRELATION OF FERRICYANIDE REDUCING ACTIVITY WITH INHIBITION BY METAL CHELATORS

PREPARATION	HILL REACTION ACTIVITY AS μM OF ELECTRONS/MG CHLOROPHYLL/HOUR		
	TO DYE		TO FERRICYANIDE
Control	652		125
Activated to ferricyanide reduction	688		403
Inhibition of Hill activity			
Molar concentration	0.0	1.6×10^{-3}	3.3×10^{-3}	6.6×10^{-3}
8-Hydroxyquinoline				
Control	0.0	0.0	20 %	92 %
Ferricyanide activated	0.0	29 %	68 %	92 %
a, a'-Dipyridyl				
Control	0.0	0.0	0.0	45 %
Ferricyanide activated	0.0	27 %	48 %	82 %

All assays were performed at 0.003 mg chlorophyll per ml. Comparisons of the effects of inhibitors were made using the 2,3',6-trichlorophenol indophenol assay of Hill activity. Activation was accomplished as in table I.

the dilution process quadrupled the rate of ferricyanide reduction but had little or no effect on the rate of pyridine nucleotide reduction.

DISCUSSION

When the spectrophotometric assay of ferricyanide reduction is used to measure the Hill activity of intact chloroplasts, a large difference in the rate of reduction of ferricyanide as compared with that of the dye 2,3',6-trichlorophenol indophenol is revealed. In both assay systems, the oxidant concentrations are sufficiently low so as to avoid their influencing the Hill reaction in the manner noted by Lumry, Spikes and Eyring (12). The reactions are zero order with respect to either dye or ferricyanide, in the concentration range used. Ferricyanide reduction by the Hill reaction of intact chloroplasts occurs at only 20 to 30 % of the rate of dye reduction.

The rate of reduction of ferricyanide is increased by sonic disruption of the chloroplasts. The rate of ferricyanide reduction can be raised to that of dye reduction by the NaCl dilution procedure described here. The dilution procedure has little or no effect on the rate of dye reduction depending on the chloroplasts, while it may increase ferricyanide reduction as much as 5-fold. A less rapid activation of ferricyanide reduction is seen with chloroplasts held in the usual storage conditions of 0.4 M sucrose buffered at pH 7.8. No gross difference could be detected in a microscopic examination of chloroplasts diluted in NaCl, compared to untreated control chloroplasts.

An analysis of the rate of the Hill reaction as a function of light intensity permits one to discern whether a limitation to Hill activity occurs at the photochemical step or at some dark reaction (12). With this analysis it can be seen that the inherent inhibition of the Hill reaction of untreated chloroplasts acts at a dark step. This inhibition is released by the NaCl dilution treatment described here.

TABLE IV

CORRELATION OF HILL ACTIVITY TO FERRICYANIDE WITH PHOTOSYNTHETIC PHOSPHORYLATION

	PREPARATION	μM Fe $(CN)_6^{-3}$ REDUCED/MG CHLOROPHYLL/HOUR	μM PO_4 ESTERIFIED/MG CHLOROPHYLL/HOUR
I	Control	172	422
	Diluted in NaCl, pH 6	800	220
	Diluted in phosphate-sucrose-KCl medium	175	515
II	Control	210	500
	Diluted in NaCl pH 6	774	57
	Diluted in phosphate-sucrose-KCl medium	227	428
III	Control	200	510
	Diluted NaCl	821	88

TABLE V

CORRELATION OF HILL ACTIVITY TO FERRICYANIDE WITH TRIPHOSPHOPYRIDINE NUCLEOTIDE REDUCTION

PREPARATION	μM Fe $(CN)_6^{-3}$ REDUCED/MG CHLOROPHYLL/HOUR	μM TPN REDUCED/MG CHLOROPHYLL/HOUR
Control	233	75.2 71.0
Ferricyanide activated	980	62.4 63.0

Activation is achieved as in table I.

The accelerating effect on ferricyanide reduction of the treatments described earlier, may be interpreted most easily as a loss of coupling between electron transport and phosphorylation (see (3) and (5)). The loss of ability to accomplish cyclic phosphorylation is consistent with this interpretation. A final proof for it is presented in the accompanying report (10).

Chloroplasts which show maximal rates of ferricyanide reduction also show increased sensitivity to metal chelators as inhibitors (table III, and (11)). This greater sensitivity could be related to a different mechanism for electron transport in uncoupled chloroplasts. Alternatively, the treatments may in some way expose previously inaccessible portions of the electron transport chain to chelating agents.

Bishop, Lumry and Spikes observed an increase in ferricyanide reducing ability of chloroplast frag-

ments in the course of aging at 24° C (6). Although it is likely that a similar interpretation will explain their observations, the leaf material (chard or sugar beet) was different, and they used chloroplasts already fragmented by a Waring blendor. Also it was necessary for the chloroplasts to be prepared under nitrogen for the effect to appear in their study.

The rate of reduction of TPN is not stimulated when ferricyanide reduction increases. Although this is not easy to explain, it is consistent with the fact that TPN reduction, under these conditions, is not stimulated by simultaneous phosphorylation (San Pietro, personal communication).

Indophenol dye reduction is much faster than ferricyanide reduction in the first place, and is not nearly as responsive to the reported treatments in the second place. The inference is that the reduction of indophenol dye is primarily by means of uncoupled electron transport, even in intact chloroplasts. This conclusion is supported by the failure to observe appreciable ATP formation while indophenol dye is being reduced (4).

A significant difference between the indophenol dyes (di- and trichloro) and ferricyanide as Hill oxidants was first observed by Witt, Moraw and Müller (15). By observing the kinetics of disappearance of a light-induced absorption increase at 512 mμ in isolated chloroplasts, they came to the conclusion that the transfer of electrons to indophenol dyes is a simpler and more rapid process than is the transfer to ferricyanide. Witt's conclusion is completely in accord with a rate limiting step in ferricyanide reduction (i.e., the coupling mechanism) which is absent or at least less important in indophenol dye reduction. It is also consistent with one possible mechanism for uncoupled electron transport, i.e., the transfer of electrons to indophenol dye from some component preceding the coupled step. This would amount to a "short circuit" of that part of the electron transport system which is coupled to phosphorylation.

It seems likely that the difference between rates of reduction of indophenol dye and of ferricyanide is one of degree of inhibition by the coupling mechanism. Thus indophenol dye reduction responds to some extent to uncoupling treatment (fig 3 and table II) and is stimulated to some extent by phosphorylating reagents (5). On the other hand some ferricyanide reduction occurs in the absence of phosphorylation even in the best chloroplast preparations now available. Finally, Spikes et al (12) observed stimulation of the Hill reaction at high concentrations of ferricyanide. This stimulation could be accounted for by an uncoupling of electron transport from the phosphorylation mechanism, due to excessive concentrations of ferricyanide.

Summary

1. Freshly prepared intact spinach chloroplasts when illuminated will reduce 2,3',6-trichlorophenol indophenol at appreciably higher rates than ferricyanide.

2. Sonication, aging, or a pretreatment of the chloroplasts involving dilution in 0.35 M NaCl at pH 6 all increase the rate of ferricyanide reduction.

3. Associated with the more rapid reduction of ferricyanide is an increased susceptibility of the chloroplast to metal chelating agents.

4. The possible relation of these effects to the uncoupling of light induced electron transport from phosphorylation is discussed.

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STABILITY OF ISOLATED CHLOROPLAST PREPARATIONS AND ITS EFFECT ON HILL REACTION MEASUREMENTS[1,2]

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Since the time of the discovery and the first characterization of the Hill reaction (13, 14) there have been many subsequent studies of this process. As might have been expected, the results obtained in these different studies are rather contradictory, so much so that it is not clear what the characteristics of the Hill reaction are. This point is well illustrated in the complete review of the older Hill reaction work found in Rabinowitch (30).

From a preliminary consideration of this problem (26) it seemed possible that the contradictions in the literature were due to the use of intact chloroplasts by some workers and fragmented chloroplasts by others. The results obtained in this study have made it clear, however, that the observed properties of a chloroplast preparation are strongly influenced by a number of factors such as the stability of the material, the species plant from which the chloroplasts were isolated and its physiological condition, and the time needed to make a measurement, to name but a few. Additional complications have been introduced by the recent finding that phosphorylation can be an integral part of the Hill reaction (2). The purpose of this paper is to fit together the pieces of this puzzle, insofar as possible, and show that one description of the Hill reaction will suffice to explain the results obtained in different studies.

MATERIALS AND METHODS

The higher plants from which chloroplasts were isolated were field grown. The leaves were picked the day they were used and were kept in a water saturated atmosphere until they were ground. Elodea sp. and Stratiotes sp. were grown in an aquarium with a layer of sand-covered soil on the bottom. The tank was illuminated by fluorescent lights with an intensity of about 100 ft-c. The lights were controlled by a timer so that a cycle of 16 hours light, 8 hours dark was maintained. *Chlorella pyrenoidosa* and *Chlamydomonas reinhardi* were grown in glass culture flasks under an atmosphere of 5 % CO_2 in air and kept at a temperature of 15 to 18° C by a water bath. Continuous illumination of about 200 ft-c was provided by fluorescent lights. In several experiments, high intensity white light (about 1500 ft-c) was provided by a 200-watt incandescent bulb, the light from which was filtered and focused by a 2-liter water-filled florence flask. The culture medium for Chlorella was that used in Emerson's laboratory (26) and the medium for Chlamydomonas was that of Granick and Sager (11) except that 20 μM/l $FeSO_4$ was used instead of ferric citrate and the micronutrients were modified A 5 and B 9. (26).

For the preparation of higher plant chloroplasts, washed turgid leaves were cut into pieces about 1 cm square. These were then ground with a mortar and pestle in buffer containing the following: 0.03 M phosphate usually at pH 7.0, 0.33 M glucose and 0.01 M KCl, unless otherwise specified. The suspension was filtered through Pyrex glass wool, centrifuged for 90 seconds at 100 G to remove whole cells and other debris, then decanted and centrifuged for 5 minutes at 900 G to bring down whole chloroplasts. This fraction was resuspended in the same buffer and centrifuged again before suspending in the final medium. Fragmented chloroplasts were removed from the whole chloroplast supernate by centrifugation at 18,000 G for 30 minutes. The supernate was decanted and discarded, and the tube rinsed with fresh buffer before resuspending the pellet. The chloroplast fragments so prepared were used without further washing.

Algal chloroplast fragments were prepared by mixing washed packed cells with kieselguhr and grinding with a mortar and pestle. The mixture was slowly resuspended in buffer and centrifuged for 5 minutes at 1000 G to remove the abrasive and whole cells. The supernate was then treated as described above for higher plant chloroplast fragments. All chloroplast preparations were carried out at temperatures between 0 and 4° C.

The photochemical activity of the chloroplasts was determined by measuring the rate of reduction of the dye 2,6, dichlorophenol indophenol, or of potassium ferricyanide. The ferricyanide was prepared immediately before use in distilled water. The concentration was measured optically assuming the molar extinction coefficient ($\log_{10}$) to be 980 at 420 mμ. The dye was purified according to the following method suggested by Dr. R. Hill: A small amount of dye, dissolved in 0.03 M phosphate buffer pH 7.1, was extracted with ether in a separatory funnel. The ether containing the acid form of the dye was filtered through double filter paper and then extracted with 0.01 M Na_2CO_3 solution until all the dye was in the aqueous phase. The dye was then salted out with NaCl, collected by filtration and air dried. The molar extinction coefficient ($\log_{10}$) of this preparation was found to be 22,400 at 610 mμ according to the method

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[2] This work was carried out while the author was a postdoctoral fellow of the National Foundation for Infantile Paralysis at the School of Biochemistry, Cambridge, England.

of Rosin (31) and was 95 % pure assuming it to be a dihydrate (N. Savage and T. Punnett, unpublished data). Because the absorption of the dye changes with pH (pK = 5.7) all changes in optical density were multiplied by the following empirically determined factors: 1.23 at pH 6.5, 1.09 at pH 6.9, 1.05 at pH 7.2, 1.00 at pH 8.0 and higher.

The photometer had 2 light paths at right angles to each other (fig 1). The light from the 250-watt mercury lamp was stabilized by a power supply (fig 2) which in turn was controlled by a photocell monitoring a portion of the output of the mercury lamp. The mercury light passed through a heat filter, and through Wratten filters which isolated the 577/579 mμ line for dye experiments or the 405 mμ line for ferricyanide experiments. The output from the measuring beam photocell was amplified by a battery operated D.C. amplifier (fig 3) and fed to a galvanometer. The light from the tungsten lamp passed through a heat filter and a red filter with a cut-off at 620 mμ. This light provided the energy for the Hill reaction and caused almost no response on the violet sensitive photocell. Galvanometer readings were taken at 15 or 20 second intervals and the final reading determined after complete reduction of the dye by ascorbic acid. The rate of change of optical density was calculated from these figures and was converted to $Q_{O_2}^{ch}$. The chlorophyll concentration in the reaction vessel, determined by Arnon's modification (3) of MacKinney's method (24), was usually between 1.0 and 8.0 μg of chlorophylls a plus b per ml.

Results

One of the first phenomena noticed in this study was that intact and fragmented chloroplasts were stable under very different conditions (27). The stability of a preparation was determined from a plot of log $Q_{O_2}^{ch}$ vs time and expressed as half-life of the photochemical activity in hours. The error in the determination of any one half-life was about 5 to 10 %, though there was often a variation of 50 to 80 % from day to day.

From the results obtained with pokeweed (*Phytolacca americana*, L.) (table I) it can be seen that whole chloroplasts in glucose (row 2 of table I) were at least 10 times more stable than the control (row 4). Fragmented chloroplasts in glucose (row 6) were only twice as stable as the control (row 8), a difference not significantly greater than error. Both fragmented and intact chloroplasts were 5 to 10 times more stable in chloride medium than in buffer alone. Furthermore, when intact chloroplasts prepared in glucose buffer were put into low osmotic pressure buffer and then returned to the glucose buffer, they no longer could be stabilized by glucose. The simplest interpretation of these results is that the high osmotic pressure glucose medium preserves the integrity of the chloroplast membrane. Once the membrane is irreversibly damaged, for example by osmotic shock, the photochemical system is inactivated rapidly unless chloride is present.

Table I

Half-life of Hill Reaction Activity (hrs) of Pokeweed Chloroplasts

Storage medium supplement	pH of storage medium 6.5	6.8	7.1	7.4	7.7	8.0	8.2
	intact chloroplasts						
1 Gluc + Cl	70	52	37	31	19	4.0	2.8
2 Glucose	73	61	200	53	5.7	2.1	1.3
3 Chloride	...	28	27	...	...	<0.6	...
4 None	...	5.5	5.5	...	...	<0.4	...
	fragmented chloroplasts						
5 Gluc + Cl	...	20	16	...	...	1.0	...
6 Glucose	...	5.5	...	...	...	<0.4	...
7 Chloride	11	16	20	23	5.0	1.4	<0.7
8 None	2.5	2.7	1.8	1.7	<0.6	<0.6	<0.6

Experiments done in 2 successive days therefore 50 % to 80 % variation between rows. Activity measured at pH 8.0. Reaction conditions as specified in Materials and Methods.

When the stability of chloroplasts from other species was determined, the results were often different. For example, chloroplasts from Good King Henry (*Chenopodium Bonus-Henricus*, L.) did not show an appreciable glucose effect (table II) although there was a pronounced chloride effect. This lack of an "intact chloroplast" effect was rather common since it was also found with pea (*Pisum sativum*, L.), and chard (*Beta vulgaris*, L.) (table III). This is consistent with the membrane hypothesis mentioned above because in these preparations, there was a great deal of starch, and starch grains are known to tear through the chloroplast membrane during centrifugation (21).

With chickweed (*Stellaria media* Cyrill), the chloroplasts were glucose stabilized only in alkaline media (pH 7.7 to 8.2) and the maximum half-life (6.0 hrs) was found at pH 7.2. In agreement with the earlier results of Hill ((15), and personal communication) it was also found that the pH of the grinding medium had to be high (ca. 7.7) in order to obtain active chloroplasts, although the chloroplasts were most stable at pH 7.2 after isolation. Recently Jagendorf and Evans (20) have shown that the beneficial effect of an alkaline grinding medium is rather general, and could be demonstrated with 5 of the 14 species they examined.

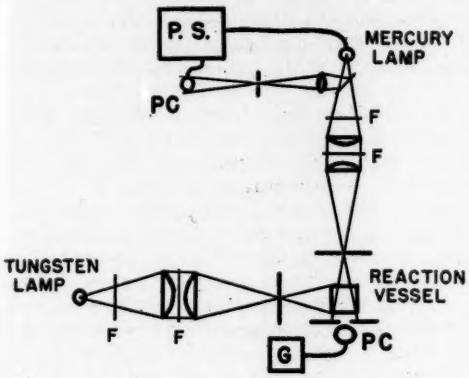
In addition to the dependence of stability on species, both the stability and the activity of chloroplasts from field grown plants were found to change during the

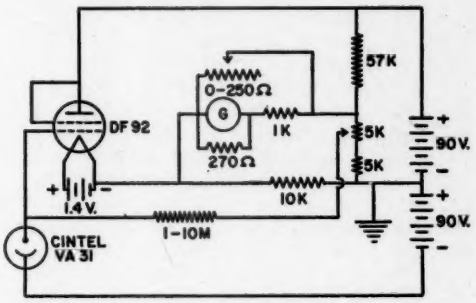
Table II

Half-life of Hill Reaction Activity (hrs) of Intact Good King Henry Chloroplasts

Storage medium supplement	pH of storage medium 7.0	7.7	8.1	8.3
1 Gluc + Cl	20	20	6.0	3.5
2 Glucose	12	6.0	3.0	...
3 Chloride	40	40	5.0	<3.0
4 None	6.5	2.5	<2.0	...

Experiments done in 1 day. Error between rows 5 % to 10 %. Activity measured at pH 7.8. Reaction conditions as specified in Materials and Methods.





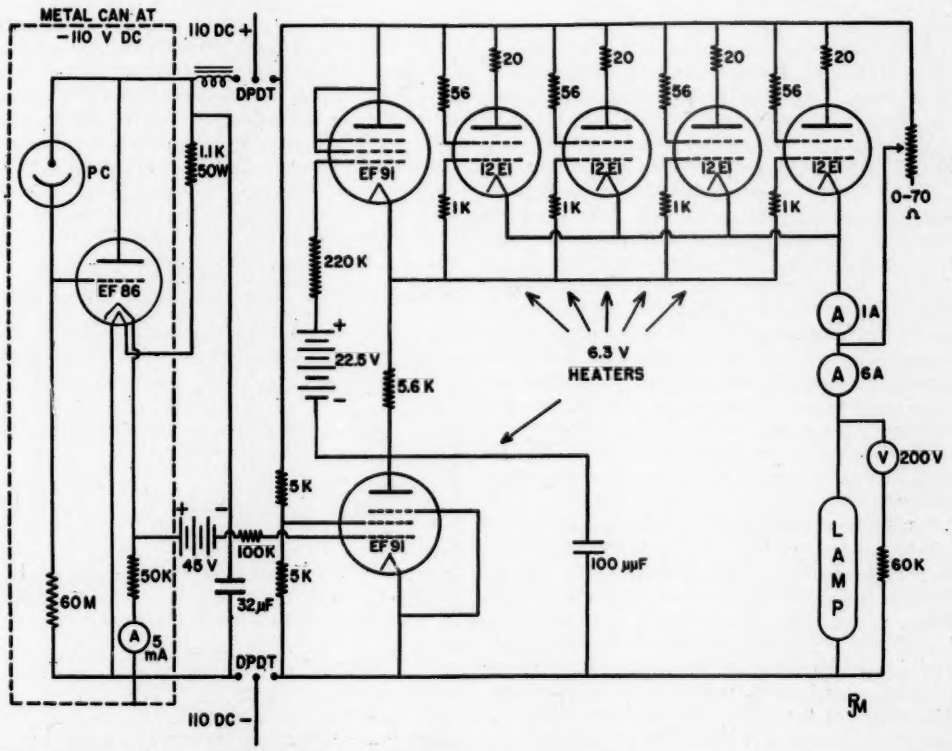


FIG. 1 (*upper left*). Diagrammatic sketch of double beam photometer.

FIG. 2 (*bottom*). Circuit diagram of feedback-stabilized D.C. power supply for mercury lamp.

FIG. 3 (*upper right*). Circuit diagram of D.C. amplifier and galvanometer for measuring beam photocell.

TABLE III

HALF-LIFE OF HILL REACTION ACTIVITY (HRS) OF INTACT CHARD CHLOROPLASTS

STORAGE MEDIUM SUPPLEMENT	pH OF STORAGE MEDIUM 6.8	7.3	7.7	8.1
1 Gluc + Cl	7.0	10	3.6	2.3
2 Glucose	5.0	6.0	2.1	1.7
3 Chloride	10	15	5.0	2.3
4 None	3.5	3.5	1.1	<0.8

Experiments done in 1 day. Variation between rows 5 % to 10%. Activity measured at pH 8.25. Reaction conditions as specified in Materials and Methods.

growing season and also seemed to depend on the growing conditions. This may explain some of the variations in activity reported by Clendenning et al (8, 9) as well as the diurnal variation observed by Hill and Scarisbrick with intact chickweed chloroplasts (16) which could not be confirmed when the experiments were repeated with fragmented pea chloroplasts ((15), and personal communication). Whether the variation in activity due to preillumination (23, 25) and the effect of red light on Hill reaction activity reported by Appleman et al (1) are also due to differences in stability remains to be seen.

The hypothesis that chloride and glucose are effective because they stabilize the material (see (34)) rather than "restore the activity" or "stimulate the Hill reaction" (12, 35) was tested in another way. Pokeweed chloroplasts, whose activity was found to be $Q_{O_2}^{ch} = 1600$ in chloride buffer, were tested for Hill reaction activity in buffer alone. After 1 minute, the reaction was stopped and chloride added to a concentration of 0.01 M. The cuvette was immediately replaced in the photometer and the reaction was resumed. The activity was 750 before the addition of chloride, and 700 afterwards. Thus there was no sign of restoring the rate to the original level, but rather a slight loss of activity possibly due to the additional mechanical agitation. Similar experiments done with glucose gave the same results. This explanation of the chloride effect differs from that of Arnon (4) only in that thermal inactivation is involved rather than photo-inactivation.

In connection with these stability experiments, it should be pointed out that the activity of the preparations was measured at "the optimum pH," usually between pH 7.7 and 8.1. These experiments were therefore comparable to those of Hill and Scarisbrick (16) and quite different from those of Warburg (35) and of Arnon (5) done at pH 6.5 to 6.8 where the photochemical activity is much lower and the stability considerably greater. This inverse relation between stability and activity was shown by the results of an experiment in which the pH curve for the Hill reaction of chard chloroplasts was measured at successive intervals (28). The values for the half-life of the Hill reaction activity as a function of pH (table IV) have been calculated from these data (fig 4) as well as the activity extrapolated to time zero.

As can be seen from figure 4, there was no optimum for this exceptionally stable preparation, and indeed there probably is no true optimum for dye reduction. The optima found in different experiments merely reflect the amount of inactivation that has occurred before the measurements are completed. This suggestion is consistent with the finding that the pH optima for the Hill reaction of chickweed, Good King Henry, pokeweed and pea chloroplasts varied from 7.7 to 8.5 from month to month, depending on the activity and the stability of the preparations. This variation in optimum did not depend on the use of fragmented vs intact chloroplasts, nor did it depend on the use of ferricyanide instead of the dye. For some reason not yet understood, we never observed the broad pH optimum at 7.1 recently found by Avron et al for very high rate ferricyanide reduction by "dilution activated" chloroplasts (7).

The greatest variation in pH optimum observed with any single species was found with chard. In early Spring the optimum was 6.9 and the activity was low ($Q_{O_2}^{ch}$ of 200 to 400), but by July the optimum rose to >8.7 and the $Q_{O_2}^{ch}$ was 2100. With chloroplasts isolated from *Chlorella pyrenoidosa* Chick, *Chlamydomonas reinhardi* Dang., 2 varieties of Elodea sp. Michx. and Stratiotes sp., the optimum was always low (ca. 6.9) as was the activity ($Q_{O_2}^{ch}$ 200 to 800). This may be due either to a species-dependent difference in the stability, or to the effect of growth under artificial light, or both. An attempt to increase the activity of the Chlorella chloroplasts by growing

TABLE IV

HALF-LIFE OF HILL REACTION ACTIVITY AS A FUNCTION OF pH*

pH	HALF-LIFE (HOURS)
6.5	130
6.6	90
6.9	120
7.2	175
7.5	95
7.8	85
7.95	48
8.1	27
8.25	23
8.4	13
8.55	9.5
8.7	7.4

* Fragmental chard chloroplasts stored in buffer containing 0.01 M KCl, 0.03 M phosphate buffer at pH 7.1.

TABLE V

EFFECT OF PREINCUBATION IN ALKALINE MEDIUM ON MANOMETRICALLY DETERMINED HILL REACTION RATES

pH OF TREATMENT		HILL REACTION RATES ($Q_{O_2}^{ch}$)	
PREINCUBATION	MEASUREMENT	CHARD	POKEWEED
6.8	6.8	735 ± 35	470 ± 25
8.1	8.1	0.0 ± 35	0.0 ± 25
6.8	8.1	200 ± 35	810 ± 25

the cells in high intensity light (ca. 1500 ft-c) proved to be unsuccessful.

The demonstration that the Hill reaction system is labile in alkaline media implies that a rapid measuring technique should be used in order to avoid interference due to inactivation. It further implies that when a slow technique such as manometry is used, it is likely that the results obtained are distorted. This may account for the fact that with one exception (17, 18), manometrically determined pH optima have been between 6.5 and 7.1. This suggestion was tested by measuring Hill reaction rates at pH 6.8 and 8.1 using the conventional manometric technique. In a 3rd vessel, the chloroplasts suspended in dilute buffer at pH 6.8 were placed in the side arm. They were tipped into the main compartment of the vessel containing buffer at pH 8.1 just before the vessels were illuminated. The rates obtained (tableV) showed that a 10-minute preincubation of the chloroplasts at an alkaline pH completely inactivated the material. On the other hand, if the chloroplasts were preincubated at pH 6.8, they retained some activity, and in the case of the more stable pokeweed showed the same pH response found photometrically.

Discussion

The results obtained in this study serve 2 purposes; they simplify greatly the interpretation of past Hill reaction studies, and they contribute to our understanding of the mechanism of the process.

One fact of primary importance for the analysis of data obtained in this and previous studies is that phosphorylation can be an integral part of the Hill reaction (2, 6, 7). As recently shown by Jagendorf and coworkers, the phosphorylation which accompanies ferricyanide reduction can be uncoupled by acid treatment (pH 6.0) or the addition of ammonium ion (19). Furthermore, dye reduction is almost independent of phosphorylation (7). For these reasons, the results

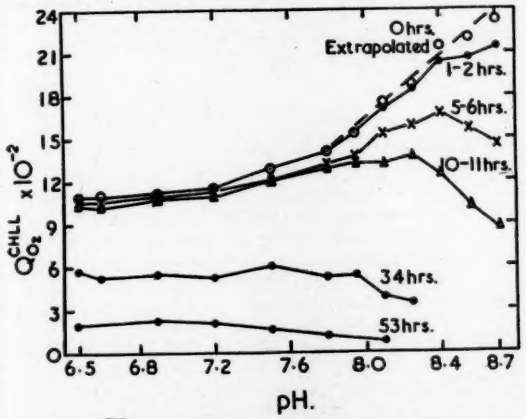


Fig. 4. Curves showing the change in the pH optimum of the Hill reaction accompanying inactivation. Curve for initial activity was determined by plotting log Q_{O2}^{ch} vs time and extrapolating to time zero.

obtained in this study did not depend on phosphorylation since either dye was used as the oxidant, or ferricyanide reduction was already uncoupled and proceeded at a high rate. This uncoupling was probably due to the low pH of the grinding buffer (22). For similar reasons, it appears that phosphorylation did not affect the results obtained in most other studies.

In retrospect it becomes clear that the contradictions concerning the chloride effect, the glucose effect, the need for intact chloroplasts, the direct reduction of ferricyanide and the pH optima are more apparent than real. The chloride effect, which is due to the stabilization of fragmented chloroplasts was found in 5 different laboratories (4, 5, 12, 27, 29, 34, 35) but not by Hill et al (10, 15, 16). This is explained by the observation that the former workers used fragmented chloroplasts or "whole chloroplasts" damaged by treatment with dilute buffer, whereas Hill et al used intact chloroplasts in their early work. The reason for the lack of a chloride effect in their later study done with Good King Henry and pea chloroplast preparations (10) is not so clear, but may have been due to stabilization of the material by the added proteins. On the other hand, the glucose effect which is due to the stabilization of intact chloroplasts was found by Hill (14) and by Holt and French (17, 18) who omitted chloride from the medium thereby insuring rapid inactivation of any fragments present. Similarly Hill's original report that only intact chloroplasts were active stems from his use of chickweed chloroplasts which lose activity extremely rapidly if damaged or fragmented in the absence of chloride. The problem of the direct reduction of ferricyanide was solved by Jagendorf and Krogman's demonstration that treatment of chloroplasts with low pH buffer (pH 6.0) uncouples phosphorylation (22). It can be seen that in all cases of direct reduction of ferricyanide, the preparation medium was unbuffered or buffered at a low pH (5, 8, 9, 29, 32, 34, 35 and this study). In the work of Hill et al (15, 16) ferricyanide reduction was almost completely blocked by an intact but inoperative phosphorylation system. The reasons for the different pH optima that have been reported have already been discussed (28). Implicit in that discussion was the concept that high activity was always associated with a high pH optimum, as demonstrated by the change in the pH response accompanying inactivation. On this basis, all low pH optima (6.5 to 7.2) could be accounted for as inactivation artifacts due to preparatory techniques or measuring techniques, or both. However, this concept must now be modified because of the recent reports that under special circumstances very high rate ferricyanide reduction (7) and cyclic phosphorylation (33) have pH optima at 7.1 with a shoulder at 7.7.

When any attempt is made to reconstruct the mechanism of the Hill reaction, it becomes clear that the system is very complex. On the basis of the results obtained in this study, it is possible to distinguish between 2 (enzymatic?) steps. The one which is normally rate-limiting in active material, enzyme a,

has the pH response shown in the top curve of figure 4 suggesting a pK at 7.5, and is relatively stable. During storage, another enzyme, enzyme b, becomes rate-limiting. Enzyme b has a pH optimum at 6.9, is stabilized by chloride ion and is relatively labile. Furthermore, the pH curves of Avron et al (7) indicate that when the phosphorylation step is limiting the rate of ferricyanide reduction, the pH curve resembles neither of the two mentioned above. This points to the existence of a third step which can be rate-limiting.

Unfortunately, this evidence deals only with the limiting steps and gives no information regarding another important problem, the question of the number of chemically different reducing sites in the chloroplast. Considering the different possibilities, it is clear that if there is only 1 reducing site, then oxidants such as ferric oxalate, the methemoglobin reducing factor and dichlorophenol-indophenol must also be uncoupling agents. On the other hand, if there are 2 or more sites, it is necessary that these oxidants react with some reducing site prior to the phosphorylation step. Furthermore, both sites must occur after the above-mentioned rate-limiting steps because the pH curves do not vary depending on the oxidant.

In conclusion, it is hoped that the resolution of many of the contradictions concerning the Hill reaction simplifies the problem of analyzing the mechanism and throws the remaining problems into sharper relief.

Summary

1. Glucose and chloride stabilize the Hill reaction system of intact and fragmented chloroplasts respectively, and do not "restore" or "stimulate" the photochemical reaction.

2. The relative stability of the Hill reaction system of intact and fragmented chloroplasts is strongly dependent on species, and probably on growth conditions.

3. The method of measuring Hill reaction activity must be rapid because at an alkaline pH where dye reduction is most rapid, the Hill reaction system is very labile.

4. The results obtained in this study are consistent with and help to resolve most of the apparent contradictions in the literature.

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This paper is affectionately dedicated to the memory of the late Robert Emerson in whose laboratory this line of investigation was begun.

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A PYRIDINE NUCLEOTIDE-CYTOCHROME C REDUCTASE ISOLATED FROM CHLOROPLASTS[1, 2]

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In 1957 Avron et al (1) extracted from spinach leaves a TPNH-diaphorase[3] which transfers electrons from TPNH to various oxidoreductive dyes but not to cytochrome c. Recently Marré et al (5, 6) isolated from chloroplasts of pea seedling a pyridine nucleotide-cytochrome c reductase which utilizes TPNH, markedly preferentially to DPNH, as an electron donor. The existence of these enzymes is of interest in view of the current concept among the investigators of photosynthesis that some cytochrome(s) as well as pyridine nucleotide(s) are involved in the mechanism of photosynthesis. Independently of these studies, we have been pursuing investigations on the oxidoreductive enzymes contained in chloroplasts, and from the chloroplasts of spinach and parsley an enzyme was isolated, which is similar to, but not identical with, that studied by Marré et al. The methods of isolation and purification as well as the properties of this enzyme form the subjects of this paper.

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[2] This research was supported by grants from the Ministry of Education and the Rockefeller Foundation.

[3] The following abbreviations are used: TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; FMN, flavine mononucleotide; FAD, flavine adenine dinucleotide; DPIP, 2,6-dichlorophenol indophenol; PCMB, p-chloromercuribenzoate; EDTA, ethylenediamine tetraacetate.

MATERIALS AND METHODS

The enzyme could be extracted from green leaves of spinach and parsley or chloroplasts isolated therefrom. To obtain chloroplasts, fresh leaves were homogenized in a Waring blendor for 3 minutes with the addition of 0.35 M NaCl solution. The homogenate was squeezed through cloth and centrifuged at 400 G for 5 minutes to remove the cell debris. The supernatant was centrifuged at 1700 G for 20 minutes, and the precipitate was collected, washed twice with 0.35 M NaCl solution by centrifugation, and used for further experiments. Two extraction procedures were employed. These led to the isolation of almost identical crude enzyme preparations.

PROCEDURE A: Fresh leaves or isolated chloroplasts (spinach) were ground in a mortar with quartz sand with the addition of chilled ammoniacal acetone-water (containing in volume percent: 98.5 acetone, 1.1 water and 0.4 ammonia) in a proportion of 85 ml per 100 g of fresh leaves or chloroplast suspension. The resulting mash was centrifuged at 1700 G for 15

minutes and the coarse precipitate was discarded. To the greenish-brown supernatant was added an equal volume of chilled ammoniacal acetone-water and the mixture was kept in an ice bath for 15 minutes. The precipitate formed was collected by filtration on a Buchner funnel and washed with weakly ammoniacal acetone-water (containing in volume percent: 74.5 acetone, 25 water and 0.15 ammonia) and then with 70 %-saturated ammonium sulfate solution (pH 8.0). The filter cake obtained was repeatedly extracted with small volumes of 0.02 M disodium phosphate solution, and the brownish-orange supernatant obtained on the final centrifugation was used as a crude enzyme preparation. The orange tint of this solution was due to contamination by the carotene protein complex previously reported from our laboratory (8).

Procedure B: Fresh leaves (250 g fresh weight) or chloroplasts (50 g fresh weight) were ground in a mortar with quartz sand and 100 ml of 0.02 M ammonium buffer (pH 8.0), to which polyethylene sorbitan monooleate (Tween 80) was added to a final concentration of 2 %. The resulting mash was centrifuged at 1700 G for 20 minutes and the green supernatant was brought to 30 %-saturation with ammonium sulfate. The solution was kept in an ice bath for 15 minutes and then centrifuged at 1700 G for 25 minutes. The supernatant was filtered on a Buchner funnel using talc as a filter aid. The brownish filtrate containing the enzyme was dialyzed in a refrigerator against 0.02 M disodium phosphate solution.

The crude enzyme preparations obtained by these 2 methods showed almost similar specific activity, but the yield was superior with Procedure B.

Purification of the Enzyme: The crude enzyme solution obtained by Procedure B was brought to 35 %-saturation with ammonium sulfate (pH 8.0) and was centrifuged to remove the brownish precipitate formed. The supernatant was made to 75 %-saturation with ammonium sulfate, and the precipitate collected by centrifugation was dissolved in 0.02 M disodium phosphate solution. The fractionation with ammonium sulfate was repeated two more times. The enzyme precipitate obtained was dissolved in a minimum volume of pure water and dialyzed in a refrigerator against 0.005 M phosphate buffer (pH 7.0). The dialysate was centrifuged at 7000 G for 15 minutes, and calcium phosphate gel was added to the clear supernatant to adsorb the enzyme. After 15 minutes, the gel was collected by centrifugation. This procedure of enzyme adsorption was repeated until the supernatant became almost colorless. The combined calcium phosphate precipitate was washed twice with pure water, and the enzyme was eluted from the gel by repeated extraction with 0.2 M disodium phosphate solution. The eluate thus obtained was dialyzed overnight in a refrigerator against 0.02 M ammonium buffer at pH 8.0 and used as the purified enzyme in the following experiments. By this purification procedure an 8- to 9-fold increase in the specific activity of the enzyme could be attained.

Activity Measurement: The enzyme activity in reducing cytochrome c in the presence of TPNH or DPNH was measured by following the increase in absorption at 550 mμ using a recording spectrophotometer. The reaction mixture used for the measurement contained: 0.02 M ammonium buffer (pH 8.0), 2.5 ml; solution of oxidized cytochrome c, 0.1 ml (final concentration: 1.7×10^{-5} M); solution of reduced pyridine nucleotide, 0.1 ml (final concentration: 1.6×10^{-5} M); enzyme preparation, 0.1 ml; and water to make a total volume of 3.0 ml. The reaction was started by adding the enzyme (or pyridine nucleotide) to the reaction mixture.

Enzymic reduction of dyes was measured by the decrease in optical density at 340 mμ (*p*-benzoquinone) or at 600 mμ (DPIP). The composition of the reaction mixture was the same as that given above except that the dye was added instead of cytochrome c. In some experiments the oxidation of pyridine nucleotide was followed by measuring the decrease in absorption at 340 mμ. All reactions were run at room temperature (around 23° C).

TPNH was prepared from TPN by reduction with glucose-6-phosphate dehydrogenase purified from the yeast (3). DPNH was prepared by the enzymic reduction of DPN with yeast alcohol dehydrogenase (7) or by sodium dithionite reduction according to LePage (4). Both TPNH and DPNH were prepared immediately before each experiment and their concentrations were determined spectrophotometrically at 340 mμ. The preparation of cytochrome c used was that from equine heart muscle, purified by the method of Keilin and Hartree (2).

Results

Stability and Susceptivity to Hydrogen Ion Concentration: The enzyme was found to be rela-

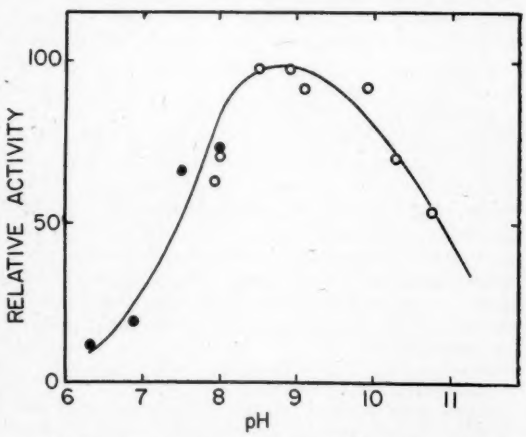

Fig. 1. pH dependence of enzyme activity. Relative activities were compared by the initial velocities of reduction of cytochrome c in the presence of TPNH. Solid circles: phosphate buffers (0.02 M) were used; open circles: ammonium buffers (0.02 M) were used.

tively stable in weakly alkaline solution, no decrease in enzyme activity being detected after several days of storage in a refrigerator under such conditions. The optimum for the reduction of cytochrome c (with TPNH as the electron donor) was found to lie at pH 8.5 to 9.0 (fig 1). All the following experiments were therefore performed at pH 8.5.

SPECIFICITY: The reduction of cytochrome c in the presence of TPNH and the enzyme as measured by the change in transmittance at 550 mμ is shown in figure 2. Under the experimental conditions adopted, the added amount of cytochrome c was completely reduced within 40 seconds. No significant change in optical density occurred when 1 of the components of the reaction was omitted, or when the enzyme had been heated at 100° C for 3 minutes. The reactions with TPNH and DPNH are compared in figure 3. As will be seen from the figure, the activity with TPNH was about 3 to 4 times as high as that with DPNH. This situation existed at different stages of purification of the enzyme (see table I).

Among the electron acceptors tested, *p*-benzoquinone and DPIP, besides cytochrome c, were found to be utilized by the enzyme, indicating a diaphorase activity of the enzyme. Also in this case the relative utilizability of TPNH and DPNH as electron donors was 3 to 4 : 1. The enzyme had no activity of catalyzing the oxidation of pyridine nucleotides by molecular oxygen.

COFACTOR REQUIREMENTS: Prolonged dialysis of crude enzyme preparation, e.g., for 3 days at 3° C against 0.005 M phosphate buffer (pH 8.0) caused a slight decrease of enzyme activity. The decrease became more marked—reducing the original activity by about 50 %—when EDTA or cyanide (10^{-5} M) was added to the dialyzing medium. In both cases the lost activity was completely recovered on the addition of molybdate (10^{-4} M) to the dialyzed enzyme (table II), indicating the role of this substance as a cofactor of the enzyme under investigation. Tungstate was also effective in this respect but with weaker efficiency. In the concentration range investigated, Ni^{++}, Ca^{++}, Zn^{++}, Mg^{++} and Fe^{++} were without any effect; other metal ions were more or less inhibitory to the enzyme activity (table II). Neither FAD nor FMN showed any restoring action upon the dialyzed enzyme in the concentration range studied (10^{-6} to 10^{-3} M) (table III). The results were also negative when FAD or FMN was added to the acid-treated preparation of the enzyme (precipitated at pH 6.0 with ammonium sulfate and redissolved in ammonium buffer of pH 8.0).

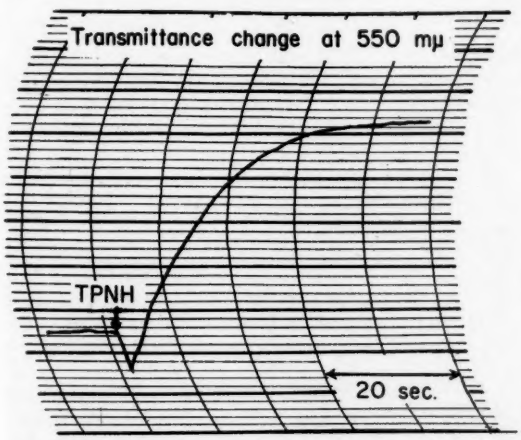

FIG. 2. Reduction of cytochrome c by the enzyme in the presence of TPNH.

TABLE I

COMPARISON OF UTILIZABILITY OF TPNH AND DPNH AS ELECTRON DONORS FOR THE ENZYME

ENZYME PREPARATION	ACCEPTOR USED	RATE OF REDUCTION* OF ACCEPTOR IN THE PRESENCE OF		RATIO
		TPNH	DPNH	
Crude	Cytochr. c.	91.0	26.0	3.5
Partially purified	Cytochr. c.	245.3	81.7	3.0
	DPIP	316.0	90.3	3.5
Purified	Cytochr. c.	839.0	227.1	3.7

* Rate of reduction of cytochrome c was measured in terms of $\Delta E_{550}/E_{273}$, where ΔE_{550} represents the change of optical density ($\times 10^3$) at 550 mμ occurring in 60 seconds, and E_{273} the optical density at 273 mμ, which was taken as a reference. The values given for DPIP are those recalculated from the rate of decrease (occurring in 60 seconds) of the optical density at 600 mμ so as to make the values correspond—in respect to the extent of electron transfer—to $\Delta E_{550}/E_{273}$.

EFFECT OF INHIBITORS: PCMB (0.4×10^{-4} M) and potassium cyanide (3×10^{-4} M) did not affect the activity of the enzyme as estimated by the reduction of cytochrome c in the presence of TPNH and molybdate. The enzyme was inhibited by EDTA, but the activity was recovered by the addition of molybdate (or tungstate) to the reaction mixture.

DISCUSSION

Considering the still insufficient purity of the enzyme preparations obtained in the present study, it may be premature to conclude decisively whether the oxidations of TPNH and DPNH were catalyzed by one and the same enzyme, or whether 2 separate enzymes, one specific towards TPNH and the other towards DPNH, were operative. However, the fairly constant ratio of 3 to 4 : 1 with respect to the (diaphorase- and cytochrome c reductase-) activities towards TPNH and DPNH at different steps of enzyme purification seems to support the former view. The above-mentioned ratio is in striking contrast to that found by Marré et al (5, 6), whose enzyme acted with TPNH

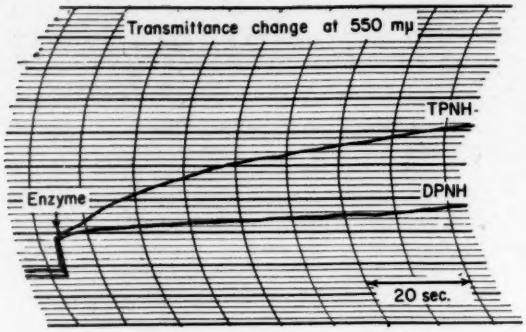

FIG. 3. Comparison of TPNH and DPNH in their effects on the reduction of cytochrome c by the enzyme. In this experiment the enzyme solution was added in the quantity of 0.01 ml (usually 0.1 ml) per 3.0 ml of the reaction mixture.

in significant preference to DPNH (about 30 : 1) in similar reactions. The enzyme studied by Avron et al (1) was claimed to be quite innert towards DPNH and cytochrome c, utilizing as acceptors only certain oxidoreductive reagents such as trichlorophenol-indophenol, ferricyanide and benzoquinone. Although our enzyme is similar to that of Marré et al in its ability to use both cytochrome c and oxidoreductive dyes as the electron acceptors, it differs from the latter in being unable to cause aerobic oxidation of pyridine nucleotides. The participation of molybdate as a cofactor is another important characteristic of our enzyme. The possible role of flavine compound(s) as a cofactor has not been clearly demonstrated in the present work, whereas positive results in this respect have been obtained with enzymes studied by Marré et al (FMN and FAD) and by Avron et al (FAD). The insensitivity of our enzyme towards inhibition by PCMB is also a point which is in marked contrast to the findings of the previous investigators. These discrepancies in the results obtained with similar materials may partly be due to the different conditions adopted in extracting the enzymes: Avron et al extracted chloroplasts with a TRIS buffer (pH 8.0) for 1 hour at room temperature, and Marré et al started the preparation with acetone powder of chloroplasts, from which the enzyme was extracted with a TRIS buffer (pH 7.3) at a lower temperature. The question as to whether or not these enzymes and ours are essentially different from each other is left open to further investigations.

TABLE II

EFFECTS OF VARIOUS METAL IONS ON THE ACTIVITY OF ENZYME WHICH HAD BEEN DIALYZED IN THE PRESENCE OF EDTA*

METAL ION ADDED**	RELATIVE ACTIVITY***
None	100
MoO_4^{--}	206
WO_4^{--}	145
Ni^{++}, Ca^{++}, Zn^{++}, Mg^{++}, Fe^{++}	97 to 103
Cd^{++}, Fe^{+++}, Al^{+++}	82 to 91
Mn^{++}, Co^{++}	52 to 70
Cu^{++}	21

* Dialyzed against 10^{-5} M EDTA for 3 days.
** Final concentration: 10^{-4} M.
*** Compared by the initial velocities of reduction of cytochrome c in the presence of TPNH.

TABLE III

EFFECTS OF FLAVINES AND MOLYBDATE ON THE ACTIVITY OF DIALYZED (EDTA-TREATED) ENZYME

ADDITIONS	RELATIVE ACTIVITY*
None	100
MoO_4^{--} (3.3×10^{-5} M)	173
FMN (2.7×10^{-6} M)	97
FMN + MoO_4^{--}	151
FAD (3.0×10^{-6} M)	100
FAD + MoO_4^{--}	173

* Compared by the initial velocities of reduction of cytochrome c in the presence of TPNH.

SUMMARY

1. From green leaves or chloroplasts of spinach and parsley a pyridine nucleotide-cytochrome c reductase was extracted either with ammoniacal acetone-water and disodium phosphate solution or with ammonium buffer of pH 8.0 containing a small amount of Tween 80. The enzyme was further purified to 8- to 9-fold increase in specific activity by fractionation with ammonium sulfate followed by adsorption on tricalcium phosphate gel.

2. The enzyme utilizes as electron donors both TPNH and DPNH and as electron acceptors cytochrome c as well as benzoquinone and 2,6-dichlorophenol-indophenol. Irrespective of the kind of electron acceptors applied, TPNH was dehydrogenated 3 to 4 times faster than DPNH. Molecular oxygen was not used as an acceptor.

3. Molybdate was found to be a cofactor of the enzyme. Flavine compounds (FMN and FAD) had no marked effect on the activity of the enzyme.

4. The results obtained were compared with those of previous investigators working with similar enzymes.

The authors wish to thank Prof. A. Takamiya of the University of Tokyo for his helpful suggestions in carrying out this research. Thanks are also due to Miss T. Oh-hama for her kind help in supplying the preparations of dehydrogenases used in this work.

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THE MECHANISM OF THE PHOTOCHEMICAL ACTIVITY OF ISOLATED CHLOROPLASTS. III. DEPENDENCE OF VELOCITY ON LIGHT INTENSITY[1,2]

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Chloroplast fragments when illuminated in suspension media containing an oxidant such as *p*-benzoquinone, potassium ferricyanide or TPN support the photoproduction of oxygen from water (1). In previous papers we have shown that the actual free-energy requirement of this type of Hill reaction is approximately independent of oxidant despite the fact that the formal requirement varies from oxidant to oxidant and is in general less than that of the complete photosynthetic process (2, 3). As a consequence, it may be concluded from free energy considerations that such Hill-reaction systems retain the remarkable efficiency of the natural process of photosynthesis. Since we have also shown that such systems yield reproducible quantitative results in rate measurements, reactions with inhibitors and the like (4), they provide a relatively simple and attractive point of attack on the photosynthetic process. Recent work, particularly by Jagendorf and his co-workers (5), has intensified interest in the Hill reaction through demonstrations of the relationship between this reaction and photophosphorylation. The later work has been carried out on chloroplast preparations which are undoubtedly more complex in chemical constitution and perhaps in structural interactions than fragments. The distinction between whole chloroplasts and fragments has not always been made in the past and, indeed, preparations have not usually been carefully characterized as to the presence or absence of intact chloroplasts. Nevertheless, it appears that chloroplast fragments, combined with necessary water-soluble cofactors and reactants, do carry out photophosphorylation just as well as intact chloroplasts. Thus, fragment preparations also provide a direct and much simplified system for investigation of this important reaction (6).

This paper confines itself to the Hill reaction of well-washed chloroplast fragments, our intention being to provide further fundamental information leading to a detailed characterization of the process. Eventually, the collection of biochemical, chemical and physical facts about photosynthesis will have to be organized on a framework of chemical kinetics if the process is to be understood in any really fundamental sense. It seems obvious that this undertaking will be very much facilitated if attention is first given to the Hill reaction, which has been shown consistently to yield simple kinetics entirely reminiscent of results obtained with chemical and enzymic systems. As will be shown in this paper, not the least fortunate appearance of simplicity is in the rate law relating velocity and light intensity. Under a very wide range of conditions, this rate law assumes a single, simple form in contradistinction to the behavior of the rate law of complete photosynthesis, which is complicated by many dependencies on extrinsic and intrinsic variables, some of which are difficult, if not impossible, to control (6). In this paper we shall establish the rate law for light intensity and provide quantitative data relative to temperature and wave length dependencies of the reaction parameters. This will be followed in the subsequent paper by a consideration of the consequences of the remarkably simple form of this law. In our treatment, we shall assume that the Hill reaction should be studied as an independent entity quite free of implications derived from observations on the total photosynthetic process in intact cells. Thus, we shall work to explain photosynthesis in terms of the Hill reaction, rather than the reverse.

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Experimental

Experimental Materials: Washed chloroplast fragment preparations were obtained by standard methods from Swiss chard leaves (Fordhook giant) or from rhubarb chard leaves. The leaves were grown in such a way as to have high intrinsic Hill-reaction activity so that as high rates of reaction as possible could be secured. It was thus generally possible to work at temperatures near zero where the loss of intrinsic activity during an experiment is usually so small as to be negligible with respect to our other errors. Careful control of blending and centrifugation procedures produced material essentially free of intact chloroplasts (3, 8). Sufficient fragments were prepared at one time for each block of statistical experiments and were stored at −35° C prior to use. Samples were thawed in exactly the same way before each experiment.

Velocity Determination: Reaction velocities were determined with conventional potentiometric or manometric techniques (3, 8, 9) using reaction mixtures which contained, in addition to chloroplast material, 0.05 to 0.10 M phosphate (total) buffer at pH 7.0, 0.167 M sucrose and the oxidant. It was previously established (4) that there are 3 ranges of oxidant effects on the Hill reaction of fragments: at very low concentrations (below 10^{-4} M) the rate falls off with oxidant concentration and is probably diffusion controlled; above 10^{-4} M and below 7×10^{-4} M, the velocity is very nearly independent of oxidant concentration; from 10^{-3} M on up, there is a complex of activating and inhibiting reactions which now appears to be associated with the decoupling of photosphorylation by oxidant (10). Independence of rate on oxidant concentration in the middle region was reaffirmed and most experiments were carried out in this region using initial oxidant concentrations of 2.5×10^{-4} M potassium ferricyanide. The uppermost region of concentrations was also investigated with 3×10^{-3} M ferricyanide, but with the use of the less precise Warburg manometric method which is more convenient for this region.

The reaction vessel was of plastic (1.57 mm along the light path and 25.4 by 12.7 mm in rectangular cross-section across it) held in a thermostated, gas-tight metal box. A vibrator-driven metal rod provided a high rate of stirring.

Previous to this study, it was the custom in our laboratory when using the potentiometric method to evaluate the amount of ferrocyanide ion produced in the reaction by calculation from potentiometric curves using the apparent half-cell potential of the ferricyanide-ferrocyanide couple provided by each curve. It was discovered in this work that such a choice of half-cell potential leads to fictitious straightening of the product-time curves thus calculated and in so doing hides thermal inactivation. Choice of the true half-cell potential appropriate to a given reaction mixture is necessary if the experiments are of such long duration that inactivation occurs. In such a case, the correct rate can be obtained by extrapolation of the slope of the product-time curve, thus correctly calculated from potentials, to zero time. The true half-cell potential can be determined using known ratios of ferricyanide to ferrocyanide ion in each kind of non-illuminated reaction mixture. The total concentrations of the ions should be at least 10^{-3} M to minimize dark reduction by the fragments. The validity of the method of correction was established by measuring reaction velocities over very short times, such that no inactivation occurred, and over long times, during which inactivation becomes so large that the rate law for inactivation can be calculated. In addition to the increase in precision obtained in this way, a further increase was secured by starting with half-reduced oxidant, for the reliability of the potential-concentration relationship is greatest in this region. Using these improvements and the fact that inactivation was usually slight, it was possible to obtain with little difficulty a standard deviation of velocity measurements of 2 % or smaller, even at low velocities.

Light-intensity Considerations: Wth the potentiometric technique, light from an incandescent lamp was passed through an interference filter (width at half-maximum passage was 50 mμ) and an infra-red filter. Intensity was regulated by voltage variation and the interpositioning of neutral density filters calibrated to better than 1 %. The intensity was maintained constant by manual regulation of the voltage and monitored continuously using a thermocouple or thermopile detector placed to the rear of the reaction cell. Absolute intensities were accurate to at least 10 %, as established by conventional methods using a National Bureau of Standards carbon-filament lamp as secondary standard, and were maintained constant within 1 %.

Because of the appreciable light absorption, even in the thinnest chloroplast suspensions, the intensity was measured as an average which can be expressed by:

$$\bar{I} = \frac{I_o - I}{\ln \frac{I_o}{I}} = \frac{I_A}{\alpha a}, \qquad (1)$$

where $\bar{I}$ = average light intensity in the suspension;
I_A = absorbed light;
I_o = light intensity passed with water in the reaction cell;
I = light intensity passed with chloroplast suspension in the cell;
α = optical density due to light absorption per unit length of chloroplast suspension; and
a = cell depth.

The total light attenuation factor α_T is obtained from measurements in an instrument such as a Beckmann DU spectrophotometer. It contains contributions from light absorption and light scattering. To a first approximation, scattering will not attenuate the local light field, so that an attenuation factor α must be defined which includes only the effect of light absorption. It is this factor determined by the methods

of this section which is used throughout this paper.

Since the sensitive elements of the thermopile or thermocouple only intercepted a small fraction of light beam transmitted through the reaction cell, it was necessary to correct the incident and transmitted light-intensity readings for divergence and scattering using a modification of the procedure of Wayrynen et al (3). A correction for the light reflected from the plastic surfaces was also necessary. The light beam divergence correction factor, f_D, was determined by placing the photo-detector in position such that it corresponded to the position of the reaction cell. With the light intensity kept constant, the galvanometer deflection was taken in this position and again with the detector in its normal position behind the cell space. The ratio of the galvanometer readings represented the divergence factor. The correction factor, f_r, for reflection losses was determined from the following expressions:

$$f_r = \frac{1}{1 - a}, \text{ where } a = \frac{\text{(gal. def. with water-filled cell)}}{2\ \text{(gal. def. without cell)}}.$$

The correction for the light lost due to scattering in the chloroplast suspension was usually calculated from galvanometer deflection readings taken with a selenium barrier layer photocell under the following conditions: 1) the cell filled with water; 2) the cell filled with the chloroplast fragment suspension; and 3) the above readings repeated with a mask over the face of the photocell in which an aperture of the same size and position of the receivers of the thermopile was cut. The scattering factor, f_s, is then

$$f_s = \frac{W_{(w/o)}}{W_{(w)}} \cdot \frac{Chl_{(w)}}{Chl_{(w/o)}},$$

where $W_{(w)}$ = galvanometer deflection with water in the cell and mask in front of the photocell;

$W_{(w/o)}$ = deflection with water in the cell and no mask in front of photocell;

$Chl_{(w)}$ = deflection with fragment suspension in the cell and mask in front of the photocell; and

$Chl_{(w/o)}$ = deflection with fragment suspension in the cell and without the mask in front of the photocell

The above procedure is based on the assumption that both the transmitted and the scattered light is picked up by the large surface of the photocell, whereas with the limited area of the aperture, the photocell picks up only the light normally received by the thermopile or thermocouple. This assumption is in keeping with the geometry of our cells, in which the depth was very much the smallest dimension. The scattering correction factor was found to vary with fragment concentration, as well as with the wave length of light, therefore it was necessary to determine the factor for every chloroplast preparation and every different light color used. Special scattering measurements are discussed in the Results section.

The reflection factor for single plastic surfaces, f_R, was found to be 1.05, whereas the divergence factor, f_D, normally varied between 1.05 and 1.10. When employing the thermocouple as the photosensitive receiver, the scattering factor, f_s, varied between 1.10 and 1.48, depending on the fragment concentration and the light color. The corrections for reflection and divergence were applied to the incident light intensity readings, whereas the divergence and scattering factors were applied to the transmitted intensity reading. It was assumed that reflected light in the fragment-filled cell was absorbed in the reaction suspension. The totally corrected average light intensity reading should thus be given by:

$$\bar{I} = \frac{f_D \cdot f_R \cdot I_o' - f_D \cdot f_S \cdot I'}{\ln \frac{f_R I_o'}{f_S I'}}, \qquad (2)$$

where I_o' and I' represent the uncorrected light intensity readings with water and reaction suspension, respectively, in the reaction cell.

In manometric experiments, the light entered from below to fall on the flat bottom of the reaction vessels. Intensity was measured with a calibrated thermocouple contained in a simulated reaction vessel and shaken through the usual orbit. Only relative intensities were needed since only the form of the rate law was required. Precision in light and velocity measurements was of the order of 2 to 5 %, depending on the magnitudes of these quantities.

Results

Macroscopic Rate Law: The equation relating average Hill-reaction velocity, v, and average light intensity, as obtained directly from the potentiometric time curve in a typical experimental situation, was indicated in an earlier work (9) to be of the form

$$v = \frac{pk\bar{I}}{\bar{I} + K}, \qquad (3)$$

in which p is determined by the fragment concentration and k and K are scale and form parameters, respectively. As a more severe test of this equation, 8 experimental velocities were determined at each of 8 light intensities. Statistical fitting to equation 3 can be carried out by an iterative least-squares method but the labor of such a treatment is justified only if the experimental errors are considerably less than those of the present investigation. It was easier and quite satisfactory to use an inverted form of equation 3 for statistical analysis:

$$\frac{\bar{I}}{v} = \frac{K}{kp} + \frac{\bar{I}}{kp}. \qquad (4)$$

Any such inverted form is suitable if proper weighting is applied. Since the standard deviation of $\bar{I}/v$ determination was found to be proportional to $\bar{I}/v$ (see table I), equation 4 with weight of $(v/\bar{I})^2$ was the obvious choice for least-squares treatment. The standard deviation of $\bar{I}$ was found to be considerably less at all intensities than that of $\bar{I}/v$, as is required for the simple least-squares method, which assumes

negligible error in the independent variable. The results, presented in table I, establish a high level of confidence in the validity of equation 4 and thus of equation 3. Calculated minus observed differences in velocity are all much smaller than the standard deviations in velocity and in most instances of the same order or less than the standard errors.

The reliability of the mean intercept (K/kp) and the mean slope (1/kp) of the regression plots according to equation 4 are measured by the standard errors (table I). Both means were found to have errors considerably less than 1 %.

CORRECTIONS FOR SUSPENSION ABSORBENCY: The results of the previous section demonstrate that the rectangular-hyperbolic rate law of equation 3 provides a highly satisfactory fit for intensity-velocity data in the macroscopic experimental situation. It is apparent, however, that unless such data can be related to the actual intensity-velocity relationship existing in any small region of constant light intensity such as might contain a few hundred chlorophyll molecules, they will not be useful for the calculation of the fundamental reaction parameters of the microscopic reaction systems. The problems associated with heterogeneous light fields such as exist in chloroplast solutions have been well discussed by Rabinowitch (11). In principle, one may extrapolate through repeated dilutions of fragment suspension to a condition of zero light absorption. In practice, it is easier to vary the cell depth along the light path, holding fragment concentration constant. Using the latter procedure, one must assume a functional form for the microscopic rate law and average this velocity expression through the reaction cell by integration. Our final choice for the microscopic law was

$$v = \frac{kI}{I + K}, \qquad (5)$$

in which v is the velocity in a local microscopic region in which the light intensity has a constant value I. A priori it might be expected that the average velocity expression derived from equation 5 by velocity averaging over the depth of the cell,

$$V_v = \frac{kp}{\mathfrak{a}a} \ln \frac{K(e^{\mathfrak{a}a} - 1) + I_A e^{\mathfrak{a}a}}{K(e^{\mathfrak{a}a} - 1) + I_A}, \qquad (6)$$

in which $\mathfrak{a}$, a, and I_A are defined as in equation 1, would provide the experimental function of velocity in terms of light intensity and optical density due to light absorption if the microscopic rate law was chosen correctly. However, as will be discussed, it was found necessary to consider also the intensity-averaged form of the integrated rate law based on the same choice of microscopic rate law,

$$V_I = \frac{kp\bar{I}}{K + \bar{I}} = \frac{kp(I_A/\mathfrak{a}a)}{K + (I_A/\mathfrak{a}a)} \qquad (7)$$

Equation 6 yields a true rectangular hyperbola at vanishingly small values of a $\mathfrak{a}$, whereas equation 7 is always a rectangular hyperbola. The data of table I have associated too large an error to allow a distinction between these equations, but at higher optical intensities the distinction becomes marked, as shown in table II, in which it can be seen that equation 7 provides a highly superior fit to the experimental data at high, as well as low, optical densities. It should be noted that the only variable used in calculating the velocities is a; K and k were the constants. Mayne has recently shown that similar verification of equations 7 and 5 results if a is held constant and $\mathfrak{a}$ is changed (12). These tests are not only adequate to distinguish between the types of averaging necessary to obtain the macroscopic rate law, but since equation 7 is satisfactory at both high and low optical densities, the microscopic rate law, equation 5, on which it is based must be a satisfactory approximation to the natural rate law to the quite high accuracy of the present data.

TABLE I

LINEAR FIT OF POTENTIOMETRIC DATA

EIGHT VELOCITY DETERMINATIONS AT EACH LIGHT INTENSITY

pH = 7.0, TEMP. 2.5° C, FERRICYANIDE CONC = 2.5×10^{-4} M, λ = 675 mμ*

Intensity**								
$\bar{I} \times 10^{-2}$ (ergs cm^{-2} sec^{-1})	1.10_4	2.36	4.37	7.08_4	9.20	23.5_5	52.0	92.0
Velocity $\frac{\text{moles ferri}}{\text{mole chlorophyll} - \text{min}}$	0.0705	0.126	0.189	0.253	0.280	0.374	0.421	0.441
Standard deviation	0.0013	0.0033	0.0032	0.0089	0.0086	0.0049	0.0064	0.0062
Standard error	0.0005	0.0012	0.0011	0.0031	0.0030	0.0017	0.0022	0.0022
Intensity/velocity $\times 10^{-3}$	1.566	1.873	2.312	2.798	3.286	6.297	12.352	20.862
Standard deviation $\times 10^{-3}$	0.0296	0.0452	0.0379	0.1002	0.1065	0.0879	0.1828	0.2957
Standard error $\times 10^{-3}$	0.0105	0.0160	0.0134	0.0354	0.0376	0.0311	0.0646	0.1046
Least-squares (Fit to equation 4)	$\frac{1}{kp} = 2.112$		$\frac{K}{kp} = 1351.5$					
Standard deviation	0.0239		24.38					
Standard error	0.0085		8.620					
Calculated v	0.0697	0.127	0.192	0.249	0.279	0.373	0.422	0.433
Calculated-observed difference	−0.0008	+0.001	+0.003	−0.004	−0.001	−0.001	+0.001	+0.002
Ratio for v, $\frac{\text{residue}}{\text{standard error}}$	−1.6	+0.8	+2.7	−1.3	−0.3	−0.6	+0.5	+0.9

* Interference filter peak.

** The standard deviation for light intensity measurements was 0.4 %.

TABLE II

COMPARISON OF V_v AND V_I WITH THE EXPERIMENTAL RATES AT DIFFERENT LIGHT INTENSITIES AND AT TWO DIFFERENT SUSPENSION THICKNESSES

ca = 1.385				ca = 2.54			
I_A* × 10^{-3}	V_v	V_I	V_{exp}	I_A* × 10^{-3}	V_v	V_I	V_{exp}
12.97	.442	.443	.441	15.80	.422	.430	.430
7.33	.420	.422	.421	8.94	.385	.401	.411
3.32	.370	.373	.374	4.05	.316	.338	.339
1.297	.278	.279	.280	1.58	.217	.234	.238
0.998	.249	.249	.253	1.215	.189	.203	.206
0.616	.195	.192	.189	0.750	.142	.149	.147
0.332	.132	.127	.126				
0.156	.0735	.0697	.0705				

Experimental conditions as in table I.
* I_A = Absorbed light in ergs/cm² —sec.
K for V_v = 590.
K for V_I = 640.

VALIDITY OF THE RECTANGULAR-HYPERBOLIC RATE LAW FOR LIGHT INTENSITY: The rectangular-hyperbolic rate law has quite generally been found to fit our velocity-light intensity data from Hill-reaction studies. Data published by Gorham and Clendenning (13) for the Hill reaction are fitted by this rate law and Burk and co-workers (14) have suggested that it is also satisfactory for photosynthesis data under some conditions (see also the discussion in Rabinowitch (11)). However, it is not possible with data of less than perfect accuracy to completely establish the validity of such an empirical analysis. We may, however, test a very great many alternative possibilities by the use of series expansions of $\frac{\bar{I}}{v}$ in powers of $\bar{I}$ since many more complicated rate laws may be approximated in this way. (For examples of photosynthesis rate laws treatable in this way see (6)). Two such expansions are sufficient:

$$\frac{\bar{I}}{v} = b + c\bar{I} + d\bar{I}^2 + e\bar{I}^3 + \ldots \quad (8)$$

$$\frac{\bar{I}}{v} = b + c\bar{I} + d\bar{I}^{-1} + e\bar{I}^{-2} + \ldots \quad (9)$$

With the use of weighted least-squares procedures, we have compared the fit of such equations to the data of table I with that of equation 7, retaining only one term beyond the linear one in these higher-order polynomials. The values of d so calculated (table III) are not only negative, but entirely insignificant by t-test procedures. Thus it is quite satisfactory to assume the validity of equations 5 and 7 for Hill-reaction data obtained at no higher precision than those of this investigation.

TEMPERATURE DEPENDENCE OF THE RATE PARAMETERS: As a consequence of the above discussion, it is possible to evaluate the rate parameters, kp and K, of the microscopic rate law from "macroscopic" velocity measurements. In earlier studies, Bishop and co-workers (8) suggested that kp/K was temperature-independent, from which they concluded that independent parameters $k/K = k_L p$ and $k = k_D p$ could be defined (see also (3)). k_L controls the rate at vanishingly small light intensities and is thus related to the light steps of the Hill reaction and to the minimum quantum requirement for this process. k_D controls the velocity at $I >> K = k_D/k_L$. Bishop also estimated the activation energy for the temperature-dependent parameter as 10 kcal. His results were based on velocity measurements at high light intensity, but he was unable to extrapolate velocities to infinite intensity. It was thus necessary to improve Bishop's results by establishing the temperature-dependence of the 2 rate parameters. Typical temperature data are plotted in figure 1 according to the inverted linear form of equation 7. Values of k_D and k_L calculated from this data by least squares are given in table IV. It is apparent that k_L has an activation energy of zero with a high degree of confidence. We may thus conclude, following the arguments of Bishop et al, that k_L has significance as a separate parameter, a result which might be expected if this parameter is dominated by temperature-independent processes such as light absorption, migration of electronic quanta and quanta-trapping through temperature-independent processes in which systems change from one electronic state to another. The activation energy for k_D obtained by least-squares fitting of the Arrhenius plot of table IV data is 13.0 ± 0.3 kcal. As would be expected, the present value is significantly higher than that reported by Bishop et al and higher than nearly all reported values, most of which were obtained without extrapolation to infinite light intensity (see (8) and (6)

TABLE III

FIT TO OTHER TRIAL EQUATIONS

TRIAL EQUATION	b	c	d
$\frac{\bar{I}}{v} = b + c\bar{I}$	14.690	2.112	...
$\frac{\bar{I}}{v} = b + c\bar{I} + d\bar{I}^2$	14.50	2.16	-4.43×10^{-4}
$\frac{\bar{I}}{v} = b + c\bar{I} + d/\bar{I}$	14.79	2.12	−0.291

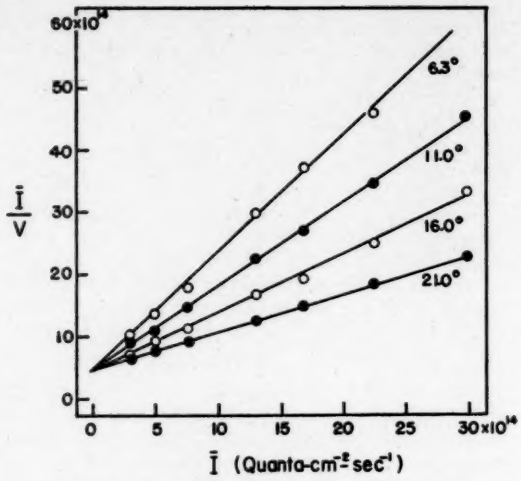
Data from table I.

TABLE IV

EFFECT OF TEMPERATURE UPON THE HILL REACTION-RATE PARAMETERS k_D AND k_L

TEMPERATURE °C	k_D	$k_L \times 10^{14}$
6.3	0.531	0.228
11.0	0.754	0.209
16.0	1.11	0.212
21.0	1.67	0.210

Experimental Conditions as in table I.



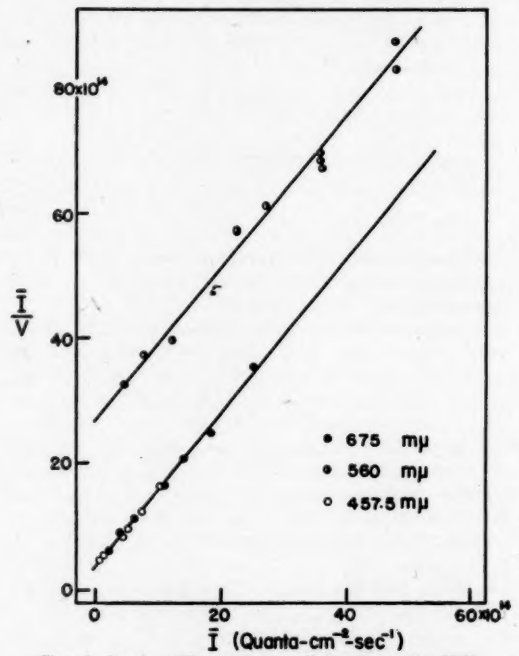


FIG. 1 (*top*). The average light intensity-Hill reaction velocity ratio plotted as a function of the average light intensity for different temperatures. This figure illustrates the contrasting temperature effects upon the slope, $1/k_D$, and the intercept, $1/k_L$, of this plot. pH = 7.0. λ = 675 mμ.

FIG. 2 (*bottom*). The average light intensity-Hill reaction velocity ratio plotted as a function of the average light intensity for 3 representative light wave lengths. 675 mμ corresponds to the region of maximum absorption of the chloroplast fragment suspension in the red region of the spectrum, 560 mμ the region of minimum absorption in the green region, and 457.5 mμ the region of maximum absorption in the blue region. Initial ferricyanide concentration = 0.00025 M. pH = 7.0. Temperature = 6.3° C.

for summary). The one exception is the value 22 kcal reported by Holt and French (15). Although reliable values of the activation entropy for k_D, $\Delta S\ddagger_D$, can not, of course, be calculated at the present time, $\Delta S\ddagger_D$ may be roughly estimated on the assumption that the rate is controlled by a fixed constituent of the fragments present in concentrations of 1 molecule per some average number of chlorophyll molecules which deliver their excitation energy to this "trapping" molecule. If we take the mean number of chlorophyll molecules to be 200, the value thus calculated is −10.9 e.u. This number is the entropy of activation corresponding to the intrinsic dark-reaction rate of 1 mole of trapping centers. It is called k_D' (see (7) for complete description). There is no reason to assume at this time that the frequency factor is other than unity.

EXPERIMENTS AT HIGH OXIDANT CONCENTRATIONS: In order to establish the form of the rate law at high (0.003 M) oxidant concentrations as well as at low, 13 carefully controlled manometric determinations of the rate law were made at the higher concentration and analyzed statistically. Although the errors inherent in the method considerably decrease the precision of these results as compared with potentiometrically determined velocities, the rate law was found to be identical in form in the 2 kinds of experiments, a confirmation of many previous observations from our laboratory. This conclusion is important since recent studies by Jagendorf and coworkers (10) suggest very strongly that the unusual behavior of the Hill reaction at high oxidant concentrations (4) is due to a decoupling by the oxidant of the photosynthetic-phosphorylation mechanism from the main sequence of Hill-reaction steps. If this indeed proves to be the case, it will be possible to conclude that the presence or absence of phosphorylation influences the values of the rate parameters but not the form of the rate law.

WAVE LENGTH DEPENDENCE OF k_D AND k_L: Lumry et al (3) obtained values for the quantum requirement of the Hill reaction using equation 4 to evaluate k_L which, on conversion to absolute absorbed light intensities, measures the minimum requirement; i.e., that which occurs at zero light intensity. Those results indicated a rather surprising increase in the minimum requirement in the region of minimum extinction coefficient for chlorophyll (560 mμ). Experience subsequent to that work suggested that the correction for scattered light may have been inadequate, particularly in the region of the spectrum in which light absorption is at a minimum. As a test of this possibility, the absolute quantum requirement was determined at 675,560 and 457.5 mμ (peak values of interference filters with a 50 mμ width at half-maximum transmission). The data are given in table V and figure 2. The earlier scattering correction was confirmed within error for blue and red illumination but not for the 560 region, where we found it necessary to use a special technique to determine the large correction. The method, due to Haxo and Blinks (16), consists in placing the chloroplast-fragment suspension against the face of a selenium barrier-

layer photocell and covering the suspension with a magnesia-coated hemisphere. A small slit in the hemisphere admits the light directly onto the suspension. Since any scattered or reflected light from the suspension will be reflected back to the photocell surface, the ratio of the photocell output with water in place of the suspension to that with the suspension in place will be the ratio of incident intensity to transmitted intensity. Comparison of this ratio with that obtained when the calibrated thermocouple was placed in the normal experimental arrangement, provided a simple calculation of the scattering factor. This factor at 560 mμ was found to be 1.48, as compared with 1.38 determined using the method described in the Experimental section. The value is considerably greater than the former value for a suspension of approximately the same fragment concentration (3).

The quantum requirements given in table V are the minimum values obtained by the extrapolation of the straight lines of figure 2 to zero light intensity and applying the necessary geometry and absorbency factors to the k_L values thus obtained. No corrections of k_L for scattering, divergence, and reflection light-losses were needed since these corrections were made on the individual light intensity readings as previously described. It will be noticed in this figure and table V that k_D (measured by slope) is essentially independent of wave length but that k_L does depend on wave length, as would be expected. It is perhaps worthy of mention that in photosynthesis, as in most other cases of biological photochemistry, the velocity of reaction at a fixed finite light intensity contains contributions from at least 2 rate processes—a light-dependent and a light-independent process—so that in the usual situation, in which the processes depend in different ways on the light intensity or on the light-wave length, a simple velocity measurement gives little, if any, useful information about the wave length dependence of this process. Thus, if it is to be useful, the concept of "action spectrum" must be more rigorously defined so that it is based on velocities extrapolated to zero light intensity. A useful action spectrum for the Hill reaction would then be a plot of properly corrected values of k_L versus wave length.

Although the quantum requirements of table V are not strictly comparable with earlier results (3), which were obtained at pH 6.3, the pH of maximum efficiency, rather than 7.0 and were thus considerably smaller than present values, the newer results do serve to correct the formerly low value at 560 mμ and to emphasize the observation that the quantum requirement at 458 mμ (and now at 560 mμ) is about 35 % higher than at 675 mμ. This latter fact may be a consequence of inefficient light absorption by accessory plant substances [4].

TABLE V

EFFECTS OF THE LIGHT WAVE LENGTH UPON THE HILL REACTION PARAMETERS k_D AND k_L

WAVE LENGTH (mμ)	k_D	$k_L \times 10^{14}$	$\sum a$	QUANTUM REQUIREMENT*
675	0.838	0.287	1.35	10.76
560	0.831	0.037	0.223	13.9
457.5	0.852	0.300	1.85	14.1

Experimental conditions except light intensity as in table I.

* $\sum a/k_L(0.436 \times 10^{14})$, quanta per oxygen produced.

DISCUSSION

RELATIONSHIP BETWEEN MACROSCOPIC AND MICROSCOPIC RATE LAWS: The rather surprising fact that the macroscopic rate law must be obtained from the microscopic law by intensity rather than velocity averaging through the reaction cell (equation 6 versus equation 7) requires explanation. Failure of the Beer-Lambert Law in our suspensions, or the existence of a time lag between exposure of the fragments to light and the appearance of product which is long with respect to the time a local fragment region requires to move from one light field to another of appreciably different intensity, would explain the observation. Since $\sum a$ was found experimentally to be a linear function of fragment concentration to considerably better than 90 % light absorption, the second explanation may be assumed tentatively to be correct. In the second case, each local reaction unit in the course of its perambulations through the rapidly stirred reaction mixture must average the light intensity. The total time between the production of product molecules, presumably oxygen, must be sufficiently long so that each unit sees approximately the same time-average light field. Gilmour (18) and Kok (19) have found that this time may be an appreciable fraction of a second but even the time of Emerson and Arnold (20), which was about 0.01 sec, is probably sufficiently long for a fragment in a rapidly stirred suspension to make a large translation in our cells. Apparently the several quanta necessary to produce 1 product molecule arrive with rates corresponding to different light intensities and thus approximately average the effect of intensity. Slow stirring (< 30 cycles per sec) produces appreciably different rates of reaction than fast stirring, though this observation must be due in part to the time for diffusional equilibration of the oxidant concentration in the neighborhood of the electrodes. Normal rates of stirring were maintained at levels (> 80 cycles per sec) such that significant changes in these rates had no effect on reaction velocities. It might be suggested that even in the absence of stirring, simple diffusional rotation of the large, optically-dense fragments would provide

[4] Studies to be reported (17) of the hydrogen-ion dependence of k_L for the fragments employed in the present potentiometric experiments show that in accordance with an earlier report from this laboratory (3), k_L has its maximum value at pH 6.3. The measurements provide, through application of a pH correction to our data obtained at pH 7.0, an estimate of the quantum requirement which we would have obtained at pH 6.3. The value thus calculated is less than 7 quanta per oxygen molecule.

some averaging of the type under discussion. However, the extreme difference in light field on 2 sides of our small fragments (about 0.2 μ in diameter) would appear to be inadequate to produce the total observed averaging.

Our experiments do suggest the validity of the second explanation based on stirring-produced translational motion and thus indicate the presence of light-intermittency effects due to stirring. Hence, most so-called steady state velocity measurements on systems of cholorplasts, chloroplast fragments, algae and photosynthetic bacteria are probably to a greater or lesser degree flashing-light experiments. Nevertheless, when such experiments are carried out in such a way that reliable extrapolation to zero and to infinite light intensity is possible, the rate parameters evaluated at these limits should exclude such effects. In the case of the reactions of chloroplast fragments when experiments which have been carried out at both very low and very high light intensities are found to fit the straight line of equation 4, we may assume that the values of k_D and k_L are very close approximations to the true steady state parameters of the microscopic as well as the macroscopic rate laws.

Summary

1. The experimental steady state, velocity-light intensity relationship for the Hill reaction of chloroplast fragments is shown to be a rectangular hyperbolic function within narrow statistical limits.

2. The rate law is analyzed in terms of the microscopic intensity-velocity relationship applying in small regions of the fragments to provide a means for evaluating fundamental rate parameters.

3. The temperature dependence of the experimental parameters is analyzed in terms of a light-step reaction parameter and a dark-step reaction parameter. The activation energy for the latter is 13.0 kcal.

4. The quantum requirement has been determined at 3 wave lengths to show that red light can be used 35 % more efficiently than green or blue light.

5. Apparent steady state studies of the Hill reaction are interpreted as flashing-light studies.

Note added in proof: In relating the rate law for the Hill reaction to the fluorescence yield-light relation, Lumry, Mayne and Spikes (Faraday Society Discussion, No. 27: Energy Transfer with Special Reference to Biological Systems, 1959) have observed that the fluorescence yield and thus presumably the local velocity at a "photosynthetic unit" for a given light intensity is independent of whether the reaction mixture is stirred or not. They conclude that the effects of stirring on measured velocity are due to the rate of equilibration of oxidant concentration at the electrode and that there is enough averaging of light intensity as a consequence of translational and rotational Brownian motion alone to explain the observed dependence of experimental velocity on average light intensity.

The paper by the above authors in the reference cited should be consulted for velocity expressions applicable to experimental situations in which the optical density is not large.

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THE MECHANISM OF THE PHOTOCHEMICAL ACTIVITY OF ISOLATED CHLOROPLASTS. V. INTERPRETATION OF THE RATE PARAMETERS [1, 2]

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In the previous paper (1) it has been shown that the steady state velocity, V, of the Hill reaction of chloroplast fragments is related to light intensity through the rectangular hyperbolic expression

$$V = \frac{pk_D k_L \bar{I}}{k_L \bar{I} + k_D} \qquad (1)$$

in which k_D and k_L are the controlling rate parameters at high and low light, respectively, p is a concentration factor and $\bar{I}$ is the average light intensity in the reaction mixture given by

$$\bar{I} = I_0(1 - e^{-\alpha n})/\alpha a \qquad (2)$$

In the latter expression, α is the attenuation factor for the light field along the axis of light propogation and a is the depth of the cell. I_0 is the intensity incident on the front face of the cell. It should be noted that α is a characteristic of a given suspension of chloroplast fragments and measures the decay in the field intensity due to absorption only since, to a first approximation in a cell of large cross section and small a, scattering will not attenuate the intensity.

It also has been shown that the "microscopic" rate law applying in the region of a few hundred chlorophyll molecules is given by

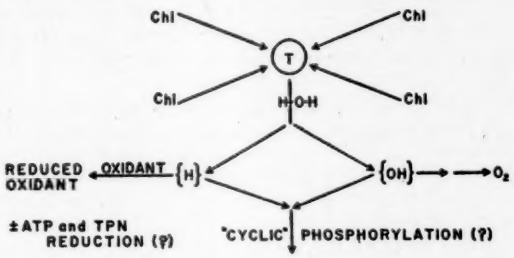
$$V = \frac{p'k_D k_L I}{k_L I + k_D} \qquad (3)$$

in which I is the constant local intensity. It is the purpose of this paper to explore the implications of the microscopic rate law using deductions based on the form of equation 3 and recent observations on the fluorescence of chloroplast fragments by Mayne et al (2, 3). In this way we shall show that a very simple picture of the reactants and reactions suffices to explain the rate law and the fluorescence observations, thus leading to a particularly useful basis for the interpretation of the rate parameters and the changes these parameters experience when experimental variables are altered.

THE MODEL: Mayne and co-workers (3) have demonstrated that the fluorescence-yield of chloroplast fragments is simply related to the steady state Hill-reaction velocity through the expression

$$[Q] = a - \frac{bV}{V_{max}} \qquad (4)$$

in which a and b are constants characteristic of a fragment preparation and Q is the natural quencher of chlorophyll fluorescence. As an aid to the present discussion, consider a modern picture of the photosynthetic apparatus in which the photophosphorylation processes of Arnon and co-workers (4) are included but an added oxidant replaces the natural oxidant.



T represents a trapping center to which an assemblage of chlorophyll molecules deliver excitation energy by resonance transfer. At T, electronic energy is converted to chemical or electrical energy, designated {T}. In the present discussion, it makes little difference whether we consider the chlorophyll molecules plus trapping center to be a "solar battery" which provides electrons and positive holes or a device to produce radical intermediates {H} and {OH}. At some points, radical chemical species must be formed, at least transiently. {T}, {H} and {OH} may be reasonably assumed to react by obligatory, catenary chains of reactions. Thus we may concern ourselves with the catenary chains of {OH} or {H} production and decomposition.

POSTULATED MECHANISMS—GENERAL: In the picture under consideration, a reaction unit consists of a trapping center with associated energy collecting chlorophyll molecules. The temperature independent rate parameter per reaction unit is k'_L. Processes which determine k'_L may be written as

$$\sum_{i=1}^{n} (C_i \xrightarrow{\alpha' I} C_i^*) \quad \text{Primary acts.}$$

$$\sum_{i=1}^{n} (C_i^* \xrightarrow{p_{ti}} T^* \quad \text{Resonance transfer to trapping center.} \qquad (5)$$

$$T^* \xrightarrow{k'_i} \{T\} \quad \text{Conversion to chemical energy.}$$

α' is the molecular extinction coefficient of the chlorophyll molecules and p_{ti} is the probability of transfer from C_i^* to T. Then, approximately,

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$$k'_L = k'_t c' \sum_{i}^{n} p_{ti} \qquad (6)$$

in which n is the mean number of chlorophyll molecules delivering to a single trapping center. The maximum quantum yield as usually determined measures $k'_t \sum_{i=1}^{n} p_{ti}$, but since some electronic quanta are lost to heat or light before trapping, the p_{ti} are not unity and further studies of fluorescence yield or fluorescence lifetime as a function of light intensity are necessary to provide the term $\sum_{i=1}^{n} p_{ti}$ and thus k'_t itself, which is the intrinsic trapping constant. Data presented by Mayne, Lumry and Spikes (3) indicate that k'_t may be as much as 150 % as large as k'_L.

The general reaction sequence for the obligatory sequence of steps in the Hill reaction is, therefore:

$$\begin{aligned}
T &\xrightarrow{k'_L I} \{T\} \\
\{T\} + E_1 &\xrightarrow{k_1} \{E_1\} + T \\
\{E_1\} + T &\xrightarrow{k_{-1}} \{T\} + E_1 \\
\{E_1\} + E_2 &\xrightarrow{k_2} \{E_2\} + E_1 \qquad (7) \\
\{E_1\} + \{E_2\} &\xrightarrow{k_{-2}} \{E_1\} + E_2 \\
&\cdots \\
\{E_n\} &\xrightarrow{k_{n+1}} \{OH\} + \{H\} + E_n \\
\{H\} + \text{oxidant} &\xrightarrow{k_{n+2}} \text{reduced oxidant} \\
\{OH\} &\xrightarrow{k_{n+3},\ \text{etc.}} O_2 .
\end{aligned}$$

{H} and {OH} need not be formed, of course, in the same elementary reaction. The E_i are fixed plant reactants and as such most are probably conserved. Fortunately, it is not necessary to consider this total scheme in our interpretation of Hill-reaction data. Indeed, we shall show that the inclusion of even a second conserved intermediate, E_1, is neither necessary nor permissible. To do this, we first employ postulated reaction sequences containing the minimum number of steps consistent with the form of the rate law and show that these do indeed satisfy the experimental facts.

MECHANISMS INVOLVING ONE CONSERVED REACTANT: Three alternative formulations are required:

$$\begin{aligned}
\text{(A)} \qquad T &\xrightarrow{k'_L I} \{T\} \\
\{T\} &\xrightarrow{k_1} T + \{OH\} + \{H\} \\
\{H\} + \text{oxidant} &\xrightarrow{\text{fast}} \text{reduced oxidant} \qquad (8) \\
\{OH\} &\xrightarrow{\text{fast}} \xrightarrow{\text{fast}} O_2 \\
[T] + [\{T\}] &= 1
\end{aligned}$$

$$\begin{aligned}
\text{(B)} \qquad T &\xrightarrow{k'_{L,1} I} \{T\}' \\
\{T\}' &\xrightarrow{k'_{L,2} I} \{T\}'' \qquad (9) \\
\{T\}'' &\xrightarrow{k_2} T + \{OH\} + \{H\} \\
\{H\} + \text{oxidant} &\xrightarrow{\text{fast}} \text{reduced oxidant} \\
\{OH\} &\xrightarrow{\text{fast}} \xrightarrow{\text{fast}} O_2 \\
[T] + [\{T\}'] + [\{T\}''] &= 1
\end{aligned}$$

$$\begin{aligned}
\text{(C)} \qquad E + C_T &\xrightarrow{k_3} T \\
T &\xrightarrow{k'_L I} \{T\} \\
\{T\} &\xrightarrow{k_4} C + \{E\} \qquad (10) \\
\{E\} &\xrightarrow{k_5} \{OH\} + \{H\} + E \\
\{H\} + \text{oxidant} &\xrightarrow{\text{fast}} \text{reduced oxidant} \\
\{OH\} &\xrightarrow{\text{fast}} \xrightarrow{\text{fast}} O_2 \\
[T] + [\{T\}] + [E] + [\{E\}] &= 1 .
\end{aligned}$$

Scheme (A) is the simplest possible, making use of only 1 plant reactant, T, in the rate-limiting steps[4]. Scheme (B) is added to cover proposals such as that of Franck, in which double-excitation is employed (5). Scheme (C) is a somewhat more detailed version of (A), including a description of T as composed of a chlorophyll molecule or other plant pigment molecule, C_T, and a plant reactant, E.

COMPARISON OF MECHANISMS INVOLVING SINGLE CONSERVED REACTANT WITH EXPERIMENT OBSERVATIONS: According to (A), if fast "finishing" steps are ignored,

$$V = \frac{m k'_L k_1 I}{k'_L I + k_1} \qquad (11)$$

so that $k_1 = k'_D : k'_D$ is the intrinsic rate of the rate-limiting process at high light for the trapping unit. m is the number of trapping units in the sample. Since T is the fluorescence quencher, equation 4 is immediately satisfied.

Similarly, from (B),

$$V = \frac{m k_2 k'_{L,2} I}{k'_{L,2} I + k_2 \left(1 + \dfrac{k'_{L,2}}{k'_{L,1}}\right)} \qquad (12)$$

$$\text{and } k_2 = k'_D,\ k'_L = \frac{k'_{L,2} k'_{L,1}}{k'_{L,2} + k'_{L,1}} .$$

[4] Note that steps of useless heat production from electronic energy need not be explicitly included in the postulated reaction sequences. Data reported by Mayne et al (3) suggest that most heat loss occurs in the pigment molecules and before quanta are trapped, rather than in the chemical step of the Hill reaction. In general, however, the inclusion of simple, first-order, heat-production processes in our mechanisms would not alter present arguments; nor need we complicate present considerations by inclusion of back reactions such as $T + \{OH\} + \{H\} \longrightarrow \{T\}$.

Either T or $\{T\}'$ may be assumed to be the fluorescence quencher, though either choice places restrictions on the relative magnitudes of $k'_{L,1}$ and $k'_{L,2}$ if equation 4 is to be satisfied. The present data give us no further information about scheme (B).

Proposal (C) yields

$$V = \frac{\text{Im}}{\left(\frac{1}{k_5} + \frac{1}{k_4} + \frac{1}{k_3}\right) I + \frac{1}{k'_L}} \qquad (13)$$

so that

$$\frac{1}{k_D'} = \frac{1}{k_5} + \frac{1}{k_4} + \frac{1}{k_3}$$

Considering T to be the quencher,

$$[T] = \frac{m \frac{1}{k'_L}}{\left(\frac{1}{k_5} + \frac{1}{k_4} + \frac{1}{k_3}\right) I + \frac{1}{k'_L}} \qquad (14)$$

and thus satisfies equation 4. The form of $\frac{1}{k_D}$ is such that any reciprocal combination of k_5, k_4 and k_3 could appear as dominant terms in $\frac{1}{k_D}$. However, the experimentally established linearity of the Arrhenius plot of $\frac{1}{k_{D\ \text{expt1}}}$ (1) suggests that if (C) is correct, 1 of the 3 elementary constants must be considerably smaller than the others and thus alone dominates k_D.

Thus, any of these 3 very simple schemes are consistent with rate-law and fluorescence-yield data. All of these probably oversimplify the situation in omitting concentration factors for "hidden" plant reactants and in concealing "hidden" elementary reactions. For example, scheme (A) may be extended to read:

(A′)
$$\begin{gathered} T \xrightarrow{k'_L I} \{T_1\} \\ \{T_1\} \rightleftarrows \{T_2\} \\ \{T_2\} \rightleftarrows \{T_3\} \\ \cdot \quad \cdot \quad \cdot \\ \cdot \quad \cdot \quad \cdot \\ \cdot \quad \cdot \quad \cdot \\ \{T_s\} \rightleftarrows \{H\} + \{OH\} + T \\ \{H\} + \text{oxidant} \longrightarrow \text{reduced oxidant} \\ \{OH\} \longrightarrow \longrightarrow O_2 \end{gathered} \qquad (15)$$

$$[T] + \sum_{i=1}^{s} [\{T_i\}] = 1$$

without violating the experimental restrictions of fluorescence and rate law (6, 7).

Postulated Mechanisms Containing Two or More Conserved Species: On the other hand, a postulated reaction scheme only slightly more complicated by the inclusion of a second conserved plant reactant E_1, such as an enzyme, is not generally acceptable. Consider—

(D)
$$\begin{gathered} T \xrightarrow{k'_L I} \{T\} \\ \{T\} + E_1 \xrightarrow{k_6} \{E_1\} + T \\ \{E_1\} + T \xrightarrow{k_{-6}} \{T\} + E \\ \{E_1\} \xrightarrow{k_7} E_1 + \{H\} + \{OH\} \\ \{H\} + \text{oxidant} \xrightarrow{\text{fast}} \text{reduced oxidant} \\ \{OH\} \xrightarrow{\text{fast}} \longrightarrow O_2 \\ [T] + [\{T\}] = 1 \\ [E_1] + [\{E_1\}] = e\ , \end{gathered} \qquad (16)$$

in which e is the total concentration of the second fixed reactant in a trapping unit. This scheme includes through proper choice of the various rate constants scheme (A), as well as those proposed for photosynthesis by Briggs (8), by Ornstein and co-workers (9) and by Kok (10). Since it contains 5 parameters, it can be made to fit steady state velocity data with high precision in many instances. Kok has regarded it as being consistent with his flashing-light studies of algal photosynthesis. However, it is not adequate to explain Gilmour's flashing-light studies of the Hill reaction (11) and we shall see that it is not generally consistent with fluorescence-yield data from the latter reaction, though in the limit when it goes over to (A) the restriction supplied by E_1 no longer applies. Its consideration here is of particular importance for 2 reasons: 1) even more complex postulated reaction systems which require the participation of additional conserved plant reactants in rate-limiting steps are at least as bad as scheme (D) and can thus be eliminated; and 2) this scheme demonstrates the type of difficulty in finding the desired class of solutions which appears with any mechanism yielding concentration time-derivatives which are not linear differential equations with constant coefficients. For an example under the second listing. One such time derivative from (D) is

$$\dot{[E_1]} = k_6 [\{T\}] [E_1] - k_{-6}[T] [\{E_1\}] - k_7 [\{E_1\}] \ . \qquad (17)$$

In this equation, as in other time derivatives, terms like $k_6 [\{T\}] [E]$ lead to unsatisfactory solutions unless $[\{T\}]$ or $[E]$ is nearly constant. Thus, if $e >> [\{E_1\}]$, $[E_1]$ could be considered constant. Then (D) reduces to (A) or (A′), since the rate is no longer limited at any time by the k_6 step. E_1 becomes a hidden reactant of (A) and the conservation of its forms has no restrictive influence on the velocity.

More generally, the steady state rate of product formation is $k_7 m [\{E_1\}]$, where $[\{E_1\}]$ is given by solutions of the equation

$$[\{E_1\}]^2 - \left(\frac{k'_L}{k_7} + \frac{k'_L}{k_6} I + e\right) [\{E_1\}] + \frac{k'_L}{k_7} e I = 0 \ . \qquad (18)$$

We have neglected k_{-6} to simplify the discussion without loss of generality. The only condition which

yields a rectangular hyperbolic rate law is the trivial solution $[\{E_1\}] << e$, which may now be expressed in a more definite manner from equation 18 as $\left(\frac{[\{E_1\}]}{e}\right)^2 << \frac{k'_L I}{k_7 e}$, a condition clearly impossible in the present case of interest, since it would produce a deviation from the simple rate law, equation 1, at experimentally attainable low light intensities. Such deviations have never been observed, even at intensities as low as 5 % of the light intensity for half-maximum velocity (12). Furthermore, the light intensity for half-maximum velocity is

$$I_{50\cdot V} = \frac{k_6 k_L + k_6 k_7 e}{k_L k_7}, \qquad (19)$$

which may be compared with that intensity for 50 % fluorescence quenching in the following simple way. If we assume T to be the quenching agent, $[\{T\}]$ must also follow a rectangular hyperbolic relation; but $[\{T\}]$ is given by solutions of

$$[\{T\}]^2 + \left(\frac{k_7}{k_6} + \frac{k_7 e}{k'_L I} - 1\right)[\{T\}] - \frac{k_7}{k_6} = 0, \qquad (20)$$

and the desired form of $[\{T\}]$ appears only if $[\{T\}]^2 < \left|\frac{k_7}{k_6}\right|$. Then

$$I_{50\cdot f} = \frac{k_6 k_7}{k'_L(k_7 - k_6 e)}. \qquad (21)$$

Clearly there is no compatibility in these results since $I_{50\cdot V} = I_{50\cdot f}$ is required by equation 4. On the other hand, if E_1 is the quencher, $I_{50\cdot V} = I_{50\cdot f}$ but the inequality $[\{E_1\}] << e$ at all $[\{E_1\}]$ cannot then be satisfied so that we conclude, in general, that scheme (D) is satisfactory only when it reduces to (A) or one of its equivalents. T must, therefore, be the quenching agent.

Conclusions:

As a consequence of the above arguments it must be supposed that either: 1) reaction systems (A), (B) or (C) are sufficient to explain the steady state Hill reaction; 2) a considerably more complicated sequence of reactions is required for this explanation but this system accidentally yields the simple rate law and fluorescence behavior under a wide range of conditions; or 3) the assumption of an obligatory reaction sequence involving conserved intermediates is incorrect. Alternative 2 is improbable. Alternative 3 may prove to be correct but appears unreasonable on the basis of available Hill-reaction data. Thus alternative 1 may be accepted as a working hypothesis to be tested in further studies; and here we should particularly like to propose flashing-light studies of the Hill reaction following the lines set forth by Gilmour (11) and by Kok (10). The value, then, of this discussion is that it supplies tentative interpretations of the experimental rate parameters in terms of definable though not yet biochemically classified plant reactants. More specifically, these results suggest that the steady state velocity of the Hill reaction of chloroplast fragments is always limited by one or another of the reactions of the trapping center T, so that past and future observations of the dependencies of k_D and k_L and fluorescence on solvent conditions, temperature, inhibitors and the like may be considered to provide information about the trapping substance or substances. It should also be stressed that the present discussion clears the way for further necessary quantitative investigations of the Hill reaction.

In conclusion we may reformulate the macroscopic experimental rate law for light intensity on the basis of the model for the trapping unit in the simplest manner suitable for future use:

$$T \xrightarrow{k_t \mathfrak{c}' n I} \{T\} \qquad (22)$$

$$\{T\} \xrightarrow{k'_D} T + \text{products}$$

$$V = \frac{k_t k'_D m a \, \mathfrak{c}' n I_o (1 - e^{-\mathfrak{c}' n m a})}{k_t \mathfrak{c}' n I_o (1 - e^{-\mathfrak{c}' n m a}) + k_D' \mathfrak{c} n m a}. \qquad (23)$$

Here m is the number of trapping centers per cc; $\mathfrak{c}'nm$ is the light attenuation factor per cm for the fragment suspension; I_o is the incident light intensity per cm^2; and $k_t = k'_t p_t$, in which p_t is the average probability that a chlorophyll a molecule will experience a resonance transfer of its quantum of excitation energy to T before loss to heat or light can occur.

Equation 1, the total velocity per cm^2 in a cell of depth a, becomes

$$V = \frac{k_t k'_D m a \mathfrak{c}' n \bar{I}}{k_t \mathfrak{c}' n \bar{I} + k'_D}, \qquad (1')$$

and if p is taken as mna, the number of chlorophyll molecules in the reaction mixture of volume $1 \times 1 \times a$ cm, the experimental quantities from equation 1′ may be interpreted as

$$k_D = \frac{k'_D}{n}$$

$$k_L = k_t \mathfrak{c}'. \qquad (24)$$

Thus the macroscopic rate parameters may be converted to the rate constants of the single average trapping unit.

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ABSORPTION SPECTRA AND RELATIVE PHOTOSTABILITY OF THE DIFFERENT FORMS OF CHLOROPHYLL IN CHLORELLA[1]

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Two or more forms of in vivo chlorophyll a with different absorption spectra appear to exist together in green plants (8, 9, 14, 16, 18, 20, 23). This paper describes the various components of the chlorophyll absorption band in the red region in Chlorella, as to their peak positions, half widths, and relative stability to light in cell homogenates.

The decline in photosynthetic efficiency at longer wave lengths within the red absorption band of chlorophyll a discovered by Emerson and Lewis (5, 6) might be attributed to coexisting active and inactive forms of chlorophyll a with the inactive form absorbing at longer wave lengths. While other explanations may be necessary to account for the supplementary light effect of Emerson, Chalmers and Cederstrand (3, 4), detailed information about the spectra and the relative photosynthetic ability of the several forms of chlorophyll a as they exist in plants is essential to an understanding of this striking phenomenon.

Evidence for the coexistence of 2 forms of chlorophyll a in green plants has been given by Krasnovsky and his co-workers (16, 18, 23) on the basis of absorption spectroscopy. A shift in the position of the red absorption peak of chlorophyll a in chloroplast suspensions caused by partial photochemical bleaching was also discovered by the Krasnovsky group (17) and attributed by them to the selective destruction of one of the two forms of chlorophyll a.

The experiments here reported were started with the hope of using this selective photobleaching as an aid in deriving the absorption curve of the more readily bleached form by subtracting the curve for the sample after partial bleaching from the original curve.

Chlorella was used for these experiments because its derivative absorption spectrum has a marked discontinuity, showing very clearly the presence of 2 overlapping components in its red absorption bands (10). Preliminary experiments showed that the chlorophyll components of Swiss chard chloroplast material bleached at an equal rate (1). Chlorella, however, gives absorption spectra of different shapes before and after bleaching, as here described.

The action spectra obtained by Haxo and Blinks (15) indicate that plants contain an active and an inactive form of chlorophyll a. The approximate absorption spectra for the active and the inactive forms have been derived (13) for *Porphyra naiadum* and for *Delesseria decipiens* from the data of Haxo and Blinks. The present paper gives more precise curves for the components of the red band of chlorophyll in Chlorella.

PROCEDURE

Chlorella pyrenoidosa Chick, Emerson, Starr Collection no. 252 was grown for 10 days in Sorokin and Myers medium (22) on a shaker over a battery of fluorescent lights. The cells were concentrated about 5 times by centrifugation and resuspension in 0.1 M phosphate buffer at pH 7. This dense suspension was forced through a needle valve (11) to liberate the chloroplasts and break them into small particles. All treatments and manipulations of the preparation after breaking the cells were carried out in the dark or in very dim light. The broken mixture was centrifuged 5 minutes at 3500 × G to remove any remaining whole cells and the larger cell fragments. The resulting supernatant, although nearly clear, shows some Tyndall effect and therefore is actually a fine colloidal suspension of cellular material the color of which is due to the chloroplast fragments. Because this preparation was so nearly clear it was possible to record its absorbance curve with adequate precision by using spectral slit widths of about 3 mμ in the Beckman recording spectrophotometer.

[1] Received April 1, 1959.

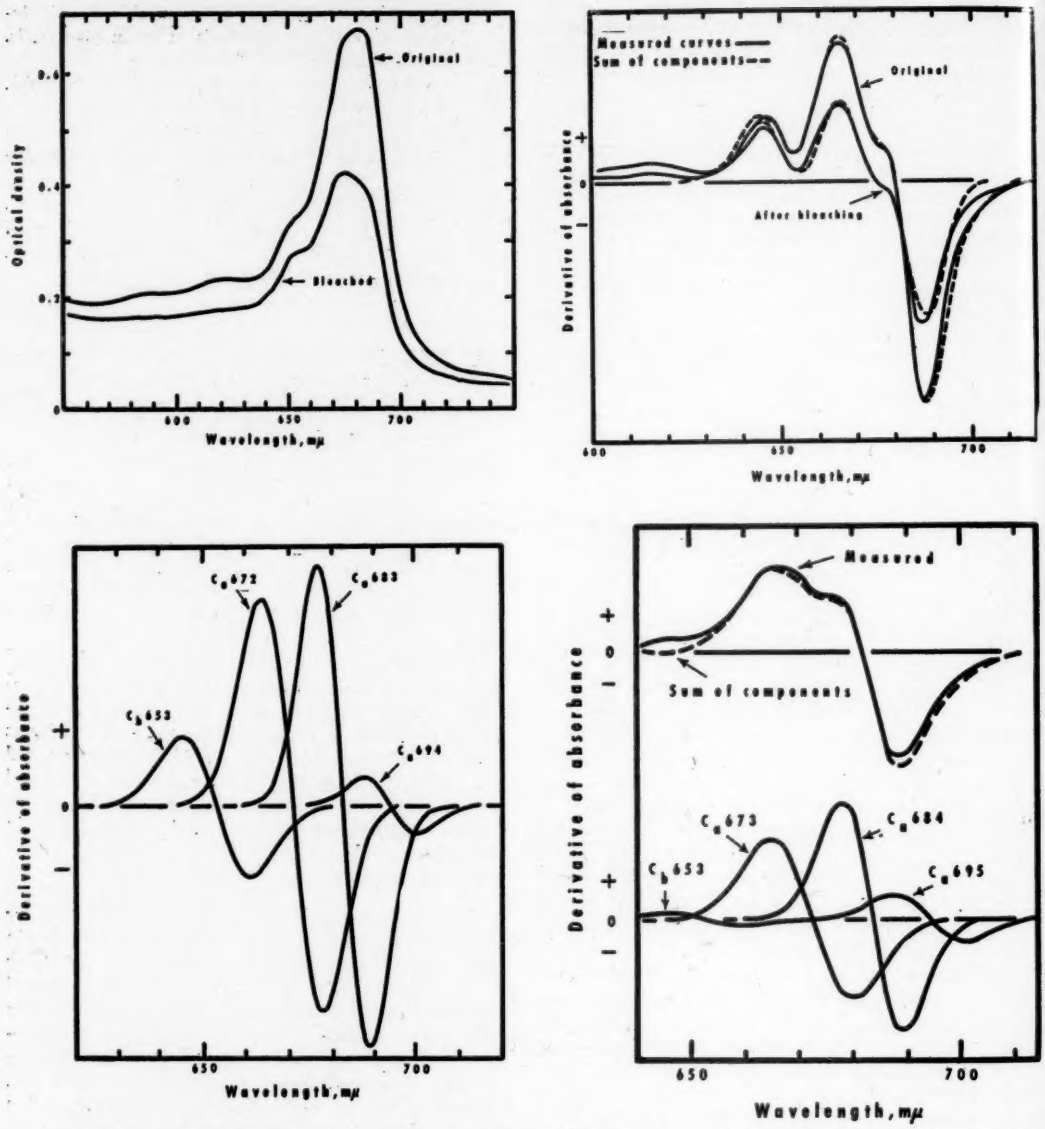

FIG. 1 (*top, left*). Absorption spectra of centrifuged Chlorella homogenate before and after partial bleaching for 15 minutes with red light.

FIG. 2 (*top, right*). Derivative spectra of the same Chlorella material as figure 1. The sums of the component curves used to fit the experimental results are given as dotted lines.

FIG. 3 (*bottom, left*). Component curves used to fit derivative spectra of the original material of figures 1 and 2. The sum of these curves is compared with the recorded derivative spectra in figure 2.

FIG. 4 (*bottom, right*). The difference between the derivative spectra of bleached and unbleached Chlorella of figure 2 and the analysis of the difference curve into its components.

Derivative spectra (7) show the band shapes much more clearly but the absorbance curves present the picture in more conventional form. Both types of measurements were used for this work. Without the derivative data the small differences between some absorbance curves, while visible, would not attract attention. To avoid the complication of carotenoid absorption the measurements were restricted to the orange and red part of the spectrum.

The partial bleaching was accomplished by illumination with a strong red light from a tungsten source through 5.5 cm of water and Corning filter no. 2418 transmitting beyond 620 mμ. With the filter removed the white light intensity at the position of the cuvette was about 100,000 ft-c.

For Experiment 1 only the derivative curves were measured before and after bleaching for 11 minutes. This bleaching time was divided into 5- and 6-minute exposures with a brief cooling period between to prevent the temperature from exceeding 30° C.

For Experiment 2 both derivative absorption spectra and the usual absorption spectra in the Beckman DK-2 were recorded with the same sample of green supernatant before and after bleaching for 10 minutes and again after an additional 5 minutes to make a total of 15 minutes of bleaching.

The experimental derivative curves were matched by summation of hypothetical components with a curve analyzer (12). The component curves were the first derivatives of the probability or Gaussian curve, $y=ae^{-bx^2}$. The curve analyzer allows us to change the horizontal or vertical scale and wave length position of each component independently and to record the individual components, as well as their sum, on the same scale as the original data. Trials were made until the sum of the components fitted the experimental curves as closely as possible.

In working with a derivative of a Gaussian curve it is convenient to express the width of the curve as the distance in wave length units between the negative and the positive peaks of the derivative curve. This value has been multiplied by 1.18, a factor which gives the width at half-height for the corresponding integral curves.

Results

Experiment 2, being more complete, will be discussed first. The absorption spectra of the supernatant from disintegrated Chlorella cells are given in figure 1 before and after 15 minutes of bleaching by red light. Figure 2 shows the derivative absorption spectra of the same preparation and also gives the summation of the component curves used to fit the experimental data. The proposed component curves are given in figure 3. By changing their size but not their position or width, these curves may also be fitted to the derivative curve for the bleached preparation as shown in figure 2.

The original hope based on Krasnovsky's results was that partial bleaching might selectively reduce the amount of only a single component thereby making it possible to determine by direct subtraction the shape of the absorption spectrum for 1 form of chlorophyll a. However, the difference spectrum between the original and the partially bleached material had a complicated shape as shown in figure 4.

This difference curve between the original and the material bleached for 15 minutes appears to consist of chlorophyll b and the 3 different chlorophyll a components given in figure 3. The sum of these components is compared with the difference curve in figure 4.

While the difference curve does not represent a single component as had been hoped, it does show that the different components present in the original preparation are not equally photosensitive. If they had been bleached proportionally, the original spectrum, that for the bleached material, and that for their difference would all have the same shape and would differ only in a scale. Furthermore, from a comparison of the curve analyses of these 3 spectra we may conclude that no components absorbing in this wave length region were produced by photo-bleaching.

The data of Experiment 1 were analyzed similarly. In addition an independent analysis by a different procedure was kindly done by Dr. Joseph E. Hayes, Jr. of the National Heart Institute. The results of these 2 curve analyses are compared in tables I and II.

The peak position and half width of the probability

Table I

Characterization of in vivo Chlorophyll Components

Peak positions, Mμ						Half-widths, Mμ					
Exp. 1			Exp 2		Selected nominal value	Exp. 1			Exp 2		Selected nominal value
Brown	Hayes		Both orig and bleached curves	Difference curve		Brown	Hayes		Both orig and bleached curves	Difference curve	
	Orig.	*Bl.*					*Orig.*	*Bl.*			
653	651	651	653	654	653	20	14	16	18	15	15
672	674	673	672	673	673	19	24	24	17	18	18
683	684	684	683	684	683	14	12	12	14	14	14
694	694	696	694	695	695	14	15	14	14	15	15

curves used to fit the data are given in table I. The agreement of peak position of the component curves selected on various occasions in this laboratory and also by Dr. Hayes for Experiment 1 are very close. In order to obtain an adequate fit the 683 component must be narrower than the 673 component. There is, however, some latitude in the possible width of the 673 component as shown in table I where either 24 or 17 mμ has been used. We prefer to keep the 2 components as nearly the same width as possible.

The relative amounts of these components, described in table I and used to fit the experimental curves, are given in table II. The relative amounts were obtained by summing the products of the vertical and horizontal distances between the peaks of each derivative component—a figure proportional to the area under each absorption band.

It is evident from the reduced percentage of the 683 component in all curves for the bleached material that the 683 component bleaches more rapidly than the 673 component, as was found by Krasnovsky. The 695 component was particularly sensitive to bleaching. The chlorophyll b peak at 653 mμ was the most stable of all.

Discussion

The derivative absorption spectra of Chlorella and of many other green algae have a plateau or shoulder near the wave length at which this curve crosses the zero line (10). (The wave length where the derivative is zero is the position of the absorption peak.) This plateau has always been observed in living Chlorella. It is present but less sharp in aqueous extracts prepared by breaking the cells in a needle-valve. An absorbance curve shows a small region of constant slope near the absorption peak corresponding to the plateau on the derivative curve.

This plateau is considered to be direct evidence for the simultaneous existence of 2 overlapping forms of chlorophyll a in vivo. The plateau is caused by the positive peak of the longer wave length component falling on or very close to the same wave length as the negative peak of the shorter wave length form. By varying the proportion of the components, the plateau can be moved along the curve. Bleaching at first destroys the longer wave length component to a greater extent than the shorter form thus causing the plateau to move toward longer wave lengths. If the proportions of the 2 components change in such a way that the plateau moves across the zero line, a shift in the absorption peak equal to the width of the plateau results. If, however, both components bleach at an equal rate, the positions of the plateau (less distinct in Swiss chard than in Chlorella) and of the absorption peak remain constant, as was found with Swiss chard chloroplasts (1). To study this situation quantitatively some sort of curve analysis is essential.

As far as we know, there is no simple physical theory that gives the universal shape of a single isolated absorption band of a dissolved pigment. Probability curves have been used successfully to fit approximately an isolated band and to analyze a complex band in terms of its individual components (24). For bands that cover an appreciable range of the spectrum, a frequency scale is preferable to a wave length scale on theoretical grounds. For narrow bands such as those of chlorophyll this extra conversion step would make too small a difference to be worth while.

Analysis by almost any assumed band shape for components is preferable to visual inspection for location of individual peak positions in complex spectra. For estimation of the band widths of individual components the shape used is more important.

In the analysis of spectra of solid particles, the distorted shape due to the flattening reported by Duysens (2) and the light scattering found by Latimer (19), even when largely avoided by attention to suitable spectrophotometry (21), might still have residual effects on band shape large enough to make the exact shape of the assumed component curves a matter of less critical importance. Analysis by probability curves is admittedly a rough-and-ready procedure, but it nevertheless appears to be the best method available giving an approximation to the actual shape of individual curves corresponding to real chemical molecules of different types in a complex mixture.

Evidence for the reality of the component at 695 mμ is not conclusive. By including this component in the curve for the original material before bleaching we get a closer fit. The 695 mμ component is not present in the bleached material. The independent curve analysis of the data from Experiment 1 also used a 695 component. In attempting to fit an intermediary (10 min) bleached curve (not illustrated by a figure, but measured during Experiment 2 and reported in table II) the 695 mμ component appeared to be present but in lesser amount than in the original. If the 695 mμ component is real, it is easily bleached. Old cultures of Euglena contain a large 695 mμ component (8). This led us to suppose that a similar component might occur in lesser amounts in other algae. However, it may also be that component curves of a different shape could give an adequate fit without a 695 component.

Table II

Relative Amounts of in vivo Chlorophyll Types in Disintegrated Chlorella Preparations

Component*	Chlorophyll b 653	Chlorophyll a 673	683	695
	Amount of each component as % of total			
Experiment 1				
Original	16	43	36	5
Bleached 11 min	23	44	29	4
Experiment 1 Hayes analysis				
Original	9	71	17	3
Bleached	15	69	13	3
Experiment 2				
Original	15	40	40	5
Bleached 10 min	18	42	37	2
Bleached 15 min	22	42	35	0
Difference, Original — Bl, 15 min	2	44	46	8

* Exact wave length and half-width used for the different curve analyses are given in table I.

The derivative absorption spectra of a large number of algae have been recorded in this laboratory. The shapes of these curves vary widely although there are similarities between closely related species. In some species the physiological age of the culture greatly influences the shape of the absorption spectrum. With Chlorella, however, the shape of the absorption curve does not change appreciably during growth. We have some evidence that these same 3 chlorophyll a component absorption spectra may be combined in various proportions to match experimental absorption curves in other algae.

The measurements here presented define the shapes of the individual component curves for chlorophylls a and b in Chlorella.

An eventual use of such curves will be to clarify the chemical nature of the different forms. However, we wish to avoid any specific interpretation of the chemical nature of the components of chlorophyll a in living plants until the number and spectral characteristics of the different chlorophyll components in various plants have been determined.

Summary

When a suspension of broken Chlorella cells, clarified by centrifugation, is partially bleached by red light, the shape of the absorption curve changes and the peak position moves towards shorter wave lengths. This shift is attributed to differing photosensitivity of the different forms of chlorophyll which have different but overlapping absorption spectra in situ. The red peaks of the individual components appear to have the following peak positions and widths in $m\mu$ at half-height: chlorophyll b: 653 (15); chlorophyll a: 673(18), 683(14), 695(15). The fraction of the total absorption due to each component and their relative sensitivity to bleaching by red light have been estimated.

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REDUCTION OF CHLOROPHYLLIDES, CHLOROPHYLLS AND CHLOROFHYLL DERIVATIVES BY SODIUM BOROHYDRIDE [1, 2]

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Sodium borohydride is a well known reducing agent. In methanol at room temperature formyl and keto groups are readily reduced to alcohol groups, while ester and carboxyl groups, carbon-carbon and carbon-nitrogen double bonds are only reduced with difficulty (2, 17). Of the groups present in chlorophyll b the C_3 formyl and the C_9 keto groups should be amenable to reduction, while the C_2 vinyl and the C_7 and C_{10} ester groups should be unaffected. Similar considerations apply to chlorophyll a and other derivatives containing formyl and keto groups.

Since previous methods of reducing pheophorbides a and b had caused either reduction of the C_2 vinyl group (6, 8) or had proceeded poorly (10), it was of interest to investigate the action of sodium borohydride upon these compounds and their magnesium salts (phyllins). Furthermore, there remained the possibility that reduction of the C_9 keto group might involve formation of an intermediate similar to the pink product of the "Krasnovsky reaction" (the reversible photobleaching of chlorophyll a by ascorbic acid in pyridine) (23).

This communication describes the preparation and the properties of the products obtained by treating methyl chlorophyllides a and b and other chlorophyll derivatives with sodium borohydride. HCl numbers, wave lengths and relative absorbancies of visible absorption maxima and minima, useful for identification purposes, are included.

MATERIALS AND METHODS

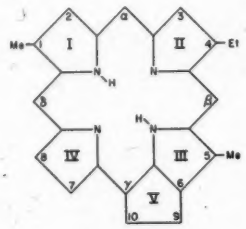
Solvents were reagent grade and were used without further purification. Sodium borohydride was obtained from Metal Hydrides Inc., Beverly, Mass. and was about 99 % pure.

Methyl chlorophyllides a and b, chlorophylls a and b, and pheoporphyrin a_5 and phylloerythrin were obtained pure by standard procedures (13, 15, 19, 20, 22, 25). "Meso" derivatives were prepared by catalytic hydrogenation of the parent compounds in acetone containing palladium black as the catalyst (14). Free acids were obtained by hydrolysis of the C_7 ester in strong hydrochloric acid for 30 minutes at room temperature (12). For the phase test, 0.1 ml of methanolic magnesium methoxide (18 mg Mg/ml) was added to 3 ml of dimethylformamide solution of pigment (18). Acid numbers were determined by the method of Willstätter and Stoll (25). Methoxyl determinations were made according to Clark (3).

Visible absorption spectra were measured by a Cary recording spectrophotometer, model 11M. Infra-red absorption spectra were measured with a Perkin-Elmer model 21 double-beam recording spectrophotometer.

The names and structural formulae of the compounds and products are those given by Fischer et al (10, 12), and are indicated in figure 1.

REDUCTION OF METHYL CHLOROPHYLLIDE b: Preliminary experiments showed that dilute methanolic solutions of chlorophyll b were readily converted by milligram amounts of sodium borohydride into a product whose color in methanol was blue-green, and whose "red" absorption maximum was at about 665 mμ. Larger amounts of sodium borohydride converted the blue-green product into one whose color was purple and whose "red" absorption maximum was at about 635 mμ.



COMPOUND	GROUPS ATTACHED TO PYRROLE β CARBON ATOMS				RING V	
	C_2	C_3	C_7	C_8	C_9	C_{10}
PHEOPHORBIDE b	Vi	CHO	X H	Me H	O	CO_2Me H
PHEOPHORBIDE b-3-METHANOL	Vi	CH_2OH	X H	Me H	O	CO_2Me H
9-OXY-DESOXO-PHEOPHORBIDE b-3-METHANOL	Vi	CH_2OH	X H	Me H	H OH	CO_2Me H
PHEOPHORBIDE a	Vi	Me	X H	Me H	O	CO_2Me H
MESO-PHEOPHORBIDE a	Et	Me	X H	Me H	O	CO_2Me H
PYROPHEOPHORBIDE a	Vi	Me	X H	Me H	O	2 H
9-OXY-DESOXO-PHEOPHORBIDE a	Vi	Me	X H	Me H	H OH	CO_2Me H
PHEOPORPHYRIN a_5	Et	Me	X	Me	O	CO_2Me H
PHYLLOERYTHRIN	Et	Me	X	Me	O	2 H
CHLORIN e_6-DIMETHYL ESTER	Vi	Me	X H	Me H	RING X ABSENT C_6–CO_2Me C_7–CH_2CO_2Me	
CHLOROPORPHYRIN e_6-DIMETHYL ESTER	Et	Me	X	Me		

FIG. 1. Structures of chlorophyll derivatives (12). (C=C, C=N and methine C–H are not indicated). Symbols: Me, (CH_3); Et, (C_2H_5); Vi, ($CH=CH_2$); X, (CH_2CH_2COOH).

[1] Received revised manuscript April 1, 1959.

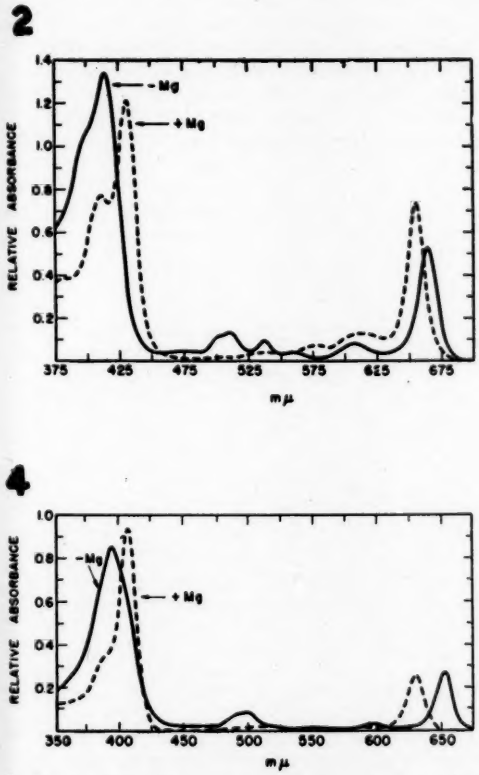
[2] Contribution from the Division of Applied Biology, National Research Council, Ottawa. Issued as N.R.C. No. 5201.

A typical preparation of the blue-green product was as follows: One-hundred ml of fresh methanolic sodium borohydride (10 mg/100 ml) were added to 36 mg of methyl chlorophyllide b dissolved in 1 ml of pyridine. After 15 minutes the pigment was transferred into ethyl ether by washing with 5 % (w/v) aqueous acid phosphate and with distilled water. It was dried and dissolved in 200 ml of 30 % (v/v) $CHCl_3$ in petroleum ether (b.p. 30 to 60° C) and then chromatographed on a sugar column using mixtures (v/v) containing 4 % $CHCl_3$ plus 1.0 or 1.4 or 1.6 % isopropanol in petroleum ether. Pigment from the main band was isolated by removing the sugar column from the glass column and by carefully cutting out the few minor contaminant bands which were present. This pigment crystallized readily from aqueous acetone.

Identification of the product as Mg-methyl pheophorbide-b-3-methanol: The C_7 ester, the C_{10} hydrogen, the C_{10} carbomethoxy, the C_9 keto and the C_2 vinyl groups were shown to be present. The evidence was as follows:

(a) C_7 ester group: Aqueous Na_2CO_3 (0.5 %, w/v) failed to extract pigment from an ether solution except after hydrolysis of the reduction product in 30 % HCl for 30 minutes at room temperature (9, 25).

(b) C_{10} hydrogen, C_{10} carbomethoxy and C_9 keto groups: The occurrence of a positive Molisch phase test confirmed the presence of all 3 groups (12). Further evidence was 1) treatment with hot, 0.5 % methanolic KOH yielded a product (acid no. 6) with a chlorin-type spectrum almost identical with that of Mg-chlorin e_5-trimethyl ester (acid no. 8) (18); 2) treatment of a pyridine solution of the magnesium-free derivative of the chlorin-type product with an equal volume of 10 % methanolic KOH effected ring closure as shown by immediate appearance of the phase test intermediate (9).



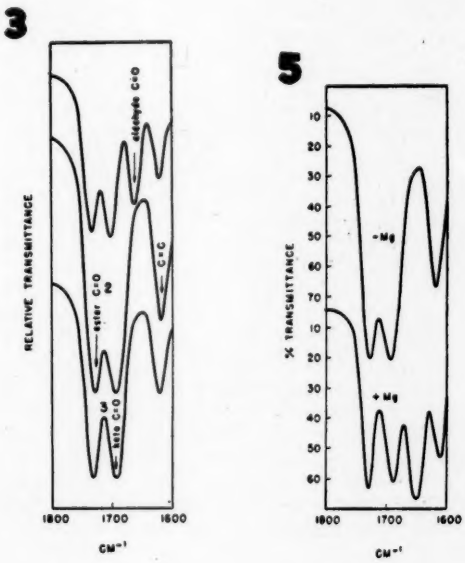


FIG. 2. Visible absorption spectra of methyl chlorophyllide b-3-methanol, and methyl pheophorbide b-3-methanol in ethyl ether.

FIG. 3. Infra-red absorption spectra of 1) Methyl pheophorbide b, 2) Methyl pheophorbide b-3-methanol and 3) Methyl pheophorbide a. Solvent: $CHCl_3$. Concentration: 5 to 6 % (w/v).

FIG. 4. Visible absorption spectra of Mg-9-oxy-desoxo-methyl pheophorbide a and 9-oxy-desoxo-methyl pheophorbide a in ethyl ether.

FIG. 5. Infra-red absorption spectra of pheophytin b-3-methanol (upper curve) and Mg-pheophytin b-3-methanol in CCl_4.

(c) C_2 vinyl group: Hydrogenation in acetone containing palladium black (14) caused a shift of the "red" absorption maximum in ether from 654 to 645 mμ. The spectrum was identical with that obtained by treating meso-methyl chlorophyllide b with sodium borohydride, in which case the absorption maximum shifted from 632 to 645 mμ. The positive phase test was retained in both products. The wave lengths of the absorption maxima of the magnesium-free product agreed well with those given earlier (8) for meso-pyropheophorbide-b-3-methanol. Such agreement was to be expected since replacement of the C_{10}-carbomethoxy group by a hydrogen atom has little or no effect on the visible spectrum.

The above findings eliminated all but the C_3 formyl group as sites of reduction. The evidence for its reduction was as follows: (a) The visible absorption spectrum, given in figure 2, was very similar to that of chlorophyll a. The wave lengths of the absorption maxima of the magnesium-free product (table I) were identical for all practical purposes with those given earlier for methyl pheophorbide b-3-methanol, obtained using aluminum isopropoxide (10). (b) The infra-red absorption spectrum, given in figure 3, showed that the formyl group was absent. The same spectrum showed that the C_9 keto group and at least 1 ester group were present (21).

Reduction of Methyl Chlorophyllide a: Two hundred and fifty ml of fresh methanolic sodium borohydride (0.3 g/250 ml) were added to 60 mg of methyl chlorophyllide a dissolved in 1 ml of pyridine. After 15 minutes at room temperature the pigment was transferred into ethyl ether and then dried. It was adsorbed on a sucrose column from 15 % $CHCl_3$ in petroleum ether. This removed 2 minor bands from the main band, and left 4 minor bands close to the top of the column. Pigment in the main band was re-chromatographed using 0.8 % isopropanol in petroleum ether which removed a small amount of yellow-green pigment. The spectra of the reduced products of methyl chlorophyllide a and methyl pheophorbide a are given in figure 4.

Identification of the product as Mg-9-oxy-desoxo-methyl pheophorbide a: By the use of some of the tests given in 1A, it was shown that the magnesium-free derivative of the reduction product contained the C_7 ester and C_2 vinyl groups. The presence of the C_{10} carbomethoxy group was shown by a) the infra-red absorption spectrum of the free acid of the reduced product contained an ester carbonyl band (21); b) the methoxyl content of the free acid was 4.8 % (calculated: 5.2 %).

The above evidence eliminated all but the C_{10} hydrogen, the C_9 keto group and the C_9–C_{10} bond as sites

Table I

Wave Lengths and Relative Absorbances of Absorption Maxima and Minima of Chlorophyll Derivatives

Compound	Maxima													
	mμ	R	mμ	R	mμ	R	mμ	R	mμ	R	mμ	R	mμ	R
Mg-methyl pheophorbide b-3-methanol	430	1.00			503 (plateau)	47.6	532	27.7	578	16.5	614	8.38	654	1.63
Mg-methyl pheophorbide a (methyl chlorophyllide a)	429	1.00			497	51.4	531	26.9	575	14.4	613	8.13	660	1.33
Methyl pheophorbide b-3-methanol	411	1.00	475	27.2	509	9.75	537	13.5	558	32.1	607	14.9	664	2.50
Methyl pheophorbide a	408	1.00	470	30.4	505	10.0	535	11.8	560	47.8	610	14.9	666	2.05
Mg-9-oxy-desoxo-methyl pheophorbide b-3-methanol	415	1.00					517	41.0			580	38.2	629	4.58
9-Oxy-desoxo-methyl-pheophorbide b-3-methanol	401	1.00	504	10.1	525	63.0	547	64.1	595	31.4	622	52.0	650	3.16
Mg-9-oxy-desoxo-methyl pheophorbide a	408	1.00					515	36.0			585	32.0	631	3.70
9-Oxy-desoxo-methyl pheophorbide a	396	1.00	500	10.0	523	70.0	549	84.0	598	34.5	622	45.0	654	3.13

Compound	Minima											
	mμ	R	mμ	R	mμ	R	mμ	R	mμ	R	mμ	R
Mg-methyl pheophorbide b-3-methanol			474	80.0	503 (plateau)	47.6	548	33.8	586	19.1	630	11.1
Mg-methyl pheophorbide a			473	88.0	507	52.7	548	36.4	588	20.7	633	13.1
Methyl pheophorbide b-3-methanol	458	33.4	486	28.6	524	24.8	550	35.0	578	55.8	626	28.2
Methyl pheophorbide a	452	39.8	481	30.8	522	26.2	546	56.0	580	66.5	630	27.5
Mg-9-oxy-desoxo-methyl pheophorbide b-3-methanol	467 (broad)	169.0					558	97.0	597	46.0		
9-Oxy-desoxo-methyl pheophorbide b-3-methanol	450	46.7			539	72.0	572	117			612	57.8
Mg-9-oxy-desoxo-methyl pheophorbide a	460 (broad)	157.0					561	100	601	37.3		
9-Oxy-desoxo-methyl pheophorbide a	465	42.6			539	96.8	571	140			615	57.2

R = Ratio of absorbance at "blue" maximum divided by the absorbance at wave length indicated.

of reaction. The presence of the C_9 secondary alcohol group, the C_{10} hydrogen and the intact C_9–C_{10} bond was shown by a) the infra-red absorption spectrum indicated that the C_9 keto group was absent; b) the phase test was negative; c) oxidation by chromic acid regenerated pheophorbide a (see section 4); d) the maxima of the visible absorption spectrum matched well those given earlier for 9-oxy-desoxo-methyl pheophorbide a (10).

REDUCTION OF PORPHYRINS: Pheoporphyrin a_5 (10 mg) was dissolved in a small volume of pyridine and added to 10 ml of sodium borohydride in methanol (5 mg/ml). After 5 minutes the pigment was transferred into ethyl ether. The reduction product was readily removed from the starting material by extraction of the ether mixture with 2 % HCl. Gradual neutralization of the dilute acid solution with $NaHCO_3$ facilitated the return of the reduction product from the aqueous layer into the ether layer. Attempts to prepare the reduced derivative of pheoporphyrin a_5 from 9-oxy-desoxo-pheophorbide a by treatment with HI in warm glacial acetic acid (13, 15) were not successful.

The spectrum of the reduction product differed from those given earlier for 9-oxy-desoxo-pheoporphyrin a_5 (5, 6) by possessing an extra weak band at about 646 mμ. The impurity responsible for this band was removed by crystallization from acetone or by extracting the ether solution with 0.2 % (w/w) aqueous HCl. The wave lengths and relative intensities of the absorption maxima of the purified product agreed well with those given by Fischer and Grassl (5) which differ by several millimicrons from the earlier data of Fischer and Hasenkamp (6). Further confirmation that the reduction product was 9-oxy-desoxo-pheoporphyrin a_5 was shown by its re-oxidation to pheoporphyrin a_5 (see section 4).

Phylloerythrin was likewise readily reduced to the 9-oxy-desoxo derivative. The spectrum of the recrystallized product was identical with that produced by the catalytic hydrogenation of phylloerythrin in formic acid containing palladium black (6, 11).

RE-OXIDATION OF THE 9-OXY-DESOXO-DERIVATIVES OF PHEOPORPHYRIN a_5 AND PHEOPHORBIDE a: Five-tenths ml of 0.5 % (w/v) chromic acid in glacial acetic acid were added to 50 ml of a benzene solution containing 3 to 5 mg of these compounds. After 15 minutes 0.5 ml more of the acid solution was added, and after 30 minutes the solution was washed well with aqueous bicarbonate and with distilled water. The benzene was removed under vacuum and the pigment was dissolved in ethyl ether. In the case of the porphyrin 2 % HCl removed the reduced products, and 10 % HCl removed pheoporphyrin a_5. With pheophorbide the acid concentrations were 8 and 12 %. The formation of pheoporphyrin a_5 and of pheophorbide a was demonstrated conclusively by their absorption spectra, by a positive phase test, by methanolysis to yield the expected open ring derivatives (4, 7, 12, 18, 24), and by the fact that borohydride reduced them back to the 9-oxy-desoxo derivatives.

TABLE II

ACID NUMBERS OF REDUCTION DERIVATIVES

COMPOUND	ACID NUMBER
Pheophytin b-3-methanol	25
Methyl pheophorbide b-3-methanol	14
9-Oxy-desoxo-pheophytin a	24
9-Oxy-desoxo-methyl pheophorbide a	8
9-Oxy-desoxo-pheophorbide a	6
9-Oxy-desoxo pheophytin b-3-methanol	21
9-Oxy-desoxo-methyl pheophorbide b-3-methanol	4

DISCUSSION

Mg-methyl pheophorbide b-3-methanol is a compound whose properties could be predicted to be very similar to those of methyl chlorophyllide a. In the red region of the spectrum it absorbs maximally in ether at 654 mμ, which distinguishes it from methyl chlorophyllide a since this absorbs maximally at 659 to 660 mμ (see table I and figure 2). The HCl number, 14 (table II), is almost identical with that of methyl pheophorbide a, 15, and is much lower than that of methyl pheophorbide b, 21 (25). A significant similarity between the 3-methanol derivative and methyl chlorophyllide a is seen in their infra-red absorption spectra between 1600 and 1800 cm^{-1}. An earlier study of the infra-red spectrum of chlorophyll a in non-polar solvents had shown the presence of an intense band at about 1650 cm^{-1}, which was absent from the spectra of chlorophyll b and of pheophytin a. The occurrence of this band is shown in figure 5 and supports the earlier hypothesis that the oxygen of the C_3 formyl group of chlorophyll b or methyl chlorophyllide b reduces the ability of magnesuim to promote enolization of the β-keto ester of ring V (21).

The shift of the "red" absorption maximum from 660 to about 630 mμ upon reduction of the C_9 keto group of methyl chlorophyllide a was reminiscent of the strong shift to about 642 mμ which occurred upon methanolysis to yield Mg-chlorin e_4-trimethyl ester (18). This strong shift has bearing upon a recent postulate that a hydrated form of ring V cycles between the enol- and keto-form during photosynthesis (1, 16). If such a hydrated form exists its spectrum in vivo should be shifted from about 685 mμ to about 655 mμ, unless the binding to a specific protein changes the spectrum and differentiates it entirely from the predominant chlorophyll a-protein complex. This follows from the fact that replacing the C_9 hydrogen atom of 9-oxy-desoxo-chlorophyll a by a hydroxyl group can have at the most only a small effect upon the parent spectrum. This is especially observable in porphyrins, e.g., the spectra of desoxo-phylloerythrin and 9-oxo-desoxo-phylloerythrin do not differ significantly (12).

It was observed (but not studied quantitatively) that the C_9 keto group is far more resistant to reduction than are formyl or keto groups at the β positions of the pyrrole nuclei. One explanation of this might be that under the conditions of the reduction, ring V existed chiefly as the enol. The addition of a small

amount of borohydride immediately converted the phase test intermediate of chlorophyll b (generated in pyridine and methanolic magnesium methoxide, 1 : 1) into one resembling that of chlorophyll a.

Summary

By means of dilute methanolic solutions of sodium borohydride a selective reduction of the C_3 formyl group of the b series of derivatives, without reduction of the C_9 keto group or other groups in the molecule can be effected. Mg-methyl pheophorbide b-3-methanol, the reduction product of methyl chlorophyllide b, has a visible absorption spectrum characteristic of the a series of pigments, but has its "red" absorption maximum shifted 5 to 6 mμ toward the "blue" relative to that of methyl chlorophyllide a. It gives a positive phase test, and has the acid number, 14. The infrared absorption spectrum of this product in non-polar solvents has a strong absorption band at about 1650 cm^{-1} just as does that of methyl chlorophyllide a. This band disappears when magnesium is removed and is not present in the spectrum of methyl chlorophyllide b. In much higher concentrations sodium borohydride effects reduction of the C_9 keto group of methyl chlorophyllide a and of the 3-methanol derivative of methyl chlorophyllide b. The reduced products have their "red" absorption maxima shifted from the 660 to the 630 mμ region. Consequently hydration of the C_9 keto group must effect a similar "blue" shift. Pheoporphyrin a_5 and phylloerythrin are also reduced to their 9-oxy-desoxo derivatives. 9-Oxy-desoxo-pheophorbide a is oxidized to pheophorbide a by dilute solutions of chromic acid in benzene. At no time during the reduction of the C_9 keto group by sodium borohydride in methanol at room temperature is an intermediate reduction product observed.

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CHLOROPLAST LIPIDS AS CARBOHYDRATE RESERVOIRS[1,2]

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While the diurnal deposition of starch within the chloroplast has long been recognized, little has been known of the photosynthate reservoirs more intimately associated with the photochemical apparatus. This paper reports recognition of chloroplast and chromatophore lipids containing significant concentrations of available carbohydrate.

A high degree of orientation of lipids within the chloroplast has been revealed by observation of positive dichroism (1, 2) when dye molecules were adsorbed in the lipid regions. Positional relationships of the photoacceptors, chlorophylls and carotenoids (3), and the chloroplast lipids almost certainly exist. Most concepts of chloroplast structure embrace these observations. It now appears that these lipids may participate in carbohydrate metabolism as well. They consist largely of galactosyl monoglycerides (4) and phosphatidyl glycerols (5). Both have the requisite long chain fatty acids and hydrophylic groups essential for orientation at lipid-pigment-protein interfaces. Their remarkable metabolic activity (6) suggests their auxiliary role as energy storage reservoirs.

MATERIALS AND METHODS

P^{32}-LABELED LEAVES AND CHLOROPLASTS: Leaves of *Tetragonia expansa* (New Zealand spinach) were illuminated during 20 hours with the petioles immersed in vials containing 1 mc radiophosphate at pH 6.8. Chloroplasts were prepared in 0.35 molar sodium chloride or 0.5 molar sucrose solutions. Aliquots of the suspension and of a similarly prepared whole leaf were analyzed for comparison with the chloroplast composition. The microscopically homogeneous suspension of cholorplasts was suspended in a minimal volume of sucrose solution and added to hot 80% ethanol. Residual pigments and lipids were extracted with hot absolute ethanol and toluene mixtures. Combined extracts were concentrated to emulsions of small volume.

PREPARATION OF CHROMATOPHORE LIPIDS: Chromatophores of light-grown *Rhodospirillum rubrum*, generously provided by Mr. Manuel Ibañez, were prepared by grinding the bacteria with alumina, eluting with phosphate buffer, and separation by differential centrifugation (7).

LIPID HYDROLYSIS: Preparations of lipids from plant sources were obtained by chloroform extraction of the concentrated aqueous emulsions and exhaustive water washing of the chloroform layer to remove nonlipid impurities. The chloroform solution and accumulated interfacial material was separated and the solvent removed in vacuo. The lipids were taken up in a minimal volume of toluene or pyridine and diluted with absolute ethanol. To aliquot portions containing lipid from measured amounts of plant material were added equal volumes of 0.2 *N* methanolic potassium hydroxide. The solution was kept at 37° C for 15 minutes (5, 8) for the base-catalyzed transesterification. An apparent pH of 11 to 12 was measured with Hydrion test paper and maintained if necessary by further alkali. After addition of a drop of water, Dowex-50 . H^+ was added to neutralize the solution. The supernatant solution and Dowex, if necessary, were applied to the origin of a chromatogram on Whatman no. 4 paper. Chromatograms were developed in phenol : water, x-direction, and in butanol : propionic acid : water, y-direction (9).

IDENTIFICATION OF LIPIDS: Although there are applicable methods for direct separation of plant lipids their analysis is greatly simplified by preliminary deacylation followed by paper chromatography of the water-soluble products. β-D-Galactosyl-1-glycerol and α-D-galactosyl-(1 → 6)-β-D-galactosyl-1-glycerol (10) were identified by cochromatography with authentic compounds kindly provided by Professor H. E. Carter. β-D(Galactosyl 6-sulfate)-1-glycerol[5] (11) was identified by cochromatography with the readily recognized compound from clover leaves. This in turn, had been identified by cochromatography with the authentic S^{35}-labeled compound from Chlorella or Scenedesmus.

Diglycerophosphate was identified by cochromatography with synthetic material. For identification of diglycerophosphate from chromatophores by neutron activation chromatography a small amount of lipids of Chlorella-P^{32} was hydrolyzed with the chromatophore lipids. The authentic diglycerophosphate-P^{32}

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[5] *Note added in proof*: The nature of the glycose in the sulfolipid is uncertain. Although analytically similar to the structure presented in figure 1, the acid stability of the glycose sulfate exceeds that of authentic galactose-6-sulfate.

was clearly recognized by the position of its radioactivity. After sufficient time for decay, the chromatogram was activated. The induced diglycerophosphate activity far exceeded the residual tracer which had been used to establish the position of the compound. Identical position was demonstrated by comparison of radiograms of the chromatogram before and after activation.

IDENTIFICATION OF SULFOLIPID IN CHLOROPLASTS OF TETRAGONIA EXPANSA: Single leaves of *T. expansa* were excised and their petioles immersed in 0.5 ml of pH 6 solution containing 1 mc of carrier-free potassium sulfate-S^{35}. After 12 hours illumination at 500 ft-c. the leaf was rinsed with water and ground with sand in sucrose solution. Chloroplasts were separated by coarse filtration and centrifugation in the usual manner. Alcohol and chloroform-alcohol extracts of the chloroplasts contained the sulfolipid-S^{35} in concentration comparable to that in Scenedesmus, 4×10^{-3} M.

In further experiments the direct isolation of the glyceryl galactoside-6-sulfate was demonstrated by application of the periodate-Schiff's reagent spray test of Buchanan et al (12). Amounts of the deacylated lipids were estimated from the relative size and intensity of the spots. Fast neutron activation of S^{32} may provide quantitative estimation of the lipid but present results provide only qualitative analysis.

NEUTRON ACTIVATION CHROMATOGRAPHIC ANALYSIS OF PHOSPHATIDES: The methods developed by Benson et al (13) were applied to the estimation of lipids derived from chromatophores of *Rhodospirillum rubrum*. Two-dimensional chromatograms on Whatman no. 4 paper were sealed in polyethylene tubes and activated with 3.6×10^{16} neutrons/cm² in the swimming pool-type reactor. After 5 to 10 days decay radioautographs were prepared on reversed single-coated x-ray films. The induced P^{32} activity was then measured by direct counting. In a typical case, 8000 cpm of P^{32} was measured in the diglycerophosphate area in a chromatogram of hydrolysate of 0.05 μl of chromatophores. The activities induced in 1μg samples of P^{31} at the borders of the chromatogram were 1000 cpm. These data indicate a concentration of 5×10^{-3} M phosphatidyl glycerol in packed chromatophores.

TABLE I

PHOSPHATIDE COMPOSITION

SOURCE	% PHOSPHATIDYL ESTER				
	CHOLINE	GLYCEROL	INOSITOL	ETHANOLAMINE	SERINE
Scenedesmus	36.1	41.2	14.2	6.1	1.0
Chromatophores of *Rhodospirillum rubrum*	30	42	15	12	
Tetragonia expansa					
leaf	59	29	12		
chloroplasts	37	34	21	6	
supernatant suspension	52	12	21	14	
Tobacco leaf	46	28	26		
Sweet clover	30.0	24.9	17.5	8.6	6.3
Barley leaf	52	22.6	14.0	1.4	
Tomato leaf	35	23	21	1.5	
Rat (total)	55	3	10	35	4

RESULTS AND DISCUSSION

Previously recognized lipids of chloroplasts appear to account for less than one fifth of those within the photochemical apparatus. The methods described have revealed a phosphatide and 3 glycolipids which occur in all photosynthetic tissues yet investigated. The galactolipids were probably included in the neutral fat of chloroplasts reported by Menke (14). The phosphatide compositions of several photosynthetic tissues and an animal tissue are given in table I. It is seen that phosphatidyl glycerol concentration is strikingly different in animal and plant tissues. It has been found in all green tissues and is the major phospholipid of isolated chromatophores of *Rhodospirillum rubrum*. It is most reliably estimated as its deacylated derivative, diglycerophosphate. P^{32}-labeling, neutron activation of P^{31} and periodate-Schiff's reagent spray have provided visible and quantitative estimation of its presence. Its concentration within

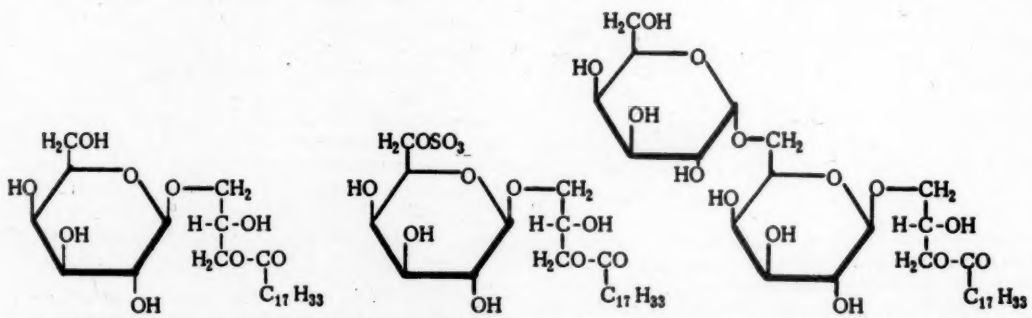

FIG. 1. The plant galactolipids. 1-*O*-oleoyl-3-(β-D-galactopyranosyl)-glycerol; 1-*O*-oleoyl-3-(β-D-galactopyranosyl 6-sulfate)-glycerol (proposed structure) and 1-*O*-oleoyl-3-(*a*-D-galactopyranosyl-(1 → 6)-β-D-galactopyranosyl)-glycerol.

Chlorella cells is about 6×10^{-3} M and within *R. rubrum* chromatophores is 5×10^{-3} M.

The chloroplast lipids of P^{32}-labeled *Tetragonia expansa* were compared to the lipids of the supernatant solution from their preparation. Chlorophyll content of the former exceeded that of the supernatant solution by 5-fold. It is seen that chloroplasts have relatively more phosphatidyl glycerol and less lecithin than the supernate. Similar relationships between chloroplast and whole leaf composition were observed.

This pool of available (15) glycerophosphate may provide a reservoir of triose phosphate and DPNH useful in phytosynthetic reactions of chloroplast. The relatively rapid glycerol turnover within this lipid in Chlorella (6) suggests its function as a metabolite as well as a structural element in the lamella.

Diglycerophosphate (10^{-2} M) → → Glycerophosphate

DPN^+ ⟶⟵ Dihydroxyacetone Phosphate (10^{-4} M) DPNH

⟶⟵ Glyceraldehyde Phosphate (10^{-5} M)

⟵⟶ Hexose Diphosphate (10^{-5} M)

The galactolipids of chloroplasts include *a*-D-galactosyl-(1 → 6)-*β*-D-galactosyl monoglyceride, *β*-D-galactosyl monoglyceride (10) and its 6-sulfate ester, figure 1. The concentrations of these lipids exceeds those of the phosphatides by 5-fold in Chlorella and red clover and similarly in spinach chloroplasts. The galactose of each is readily labeled during photosynthesis in $C^{14}O_2$ (4, 6). While quantitative methods for their estimation are not yet available it would seem reasonable to anticipate that the concentration of the galactose moieties in chloroplast lipids varies with metabolic status and constitutes a major carbohydrate reservoir in the chloroplast. Functional relationships of these *β*-galactosides to the *a*-galactosyl-2-glycerol of red algae (16) is uncertain.

The plant sulfolipid is the most concentrated anionic lipid in chloroplasts. The sulfate ester group provides a much stronger anionic group than does the phosphate diester group of the glycerolphosphatides. It is conceivable that the physical properties of the sulfolipid could lead to laminar electrostatic fields at lipid-protein interfaces with function useful in photosynthesis. Oriented lipid layers as probably occur within the plastid might act as metabolically-controlled ion exchange membranes as well. Observation of ion exchange properties of ionic lipid films should provide interesting information and allow further proposals for their function in chloroplasts.

Summary

The galactolipids, phospholipids and sulfolipids of the photosynthetic cell appear to be concentrated within the chloroplast and chromatophore. Of these compounds mono- and digalactosyl monoglycerides constitute the predominant fraction.

The photosynthetic apparatus contains the 2 anionic lipids, phosphatidyl glycerol and the plant sulfolipid, a glycosyl sulfate lipid in concentrations approximating 10^{-2} M.

The function of the lipid galactose and glycerophosphate as reservoirs of carbohydrate within the lamellar photosynthetic structures is discussed.

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FACTORS AFFECTING LIGHT INDUCED FIXATION OF CARBON DIOXIDE BY ISOLATED SPINACH CHLOROPLASTS [1, 2]

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Fager (9) and Arnon et al (3) made the fundamental observation that isolated chloroplast material could assimilate CO_2 and that the incorporation of CO_2 was enhanced by light. Since the products formed by the chloroplasts during their exposure to tracer CO_2 (1) were similar to those described by Bassham and Calvin (8) for intact cells, the general concept was presented that the extracellular metabolism of CO_2 by the isolated particle retains the principal features of the intact system. The similarity between the tracer pattern of the starch glucose formed by the intact cells and isolated chloroplasts of spinach was taken as further evidence by Gibbs and Cynkin (12) that the isolated structure possesses a typical photosynthetic carbon cycle. This paper reports experiments on light induced $C^{14}O_2$ assimilation by isolated spinach chloroplasts and the effect of pH, various classical inhibitors and other factors on this process. A preliminary report on part of this investigation has appeared (11).

MATERIALS AND METHODS

PREPARATION OF CHLOROPLASTS: Leaves obtained from various sources were washed with distilled water and placed in an ice slush for 0.5 hour. The midribs were removed from the turgid leaves and the leaf blades were dried with blotting paper and weighed. The leaf blades were cut into small pieces. In some experiments, spinach plants grown in hydroponic solution or in pots with a light period of 16 hours and a temperature of 16 to 21° C were used. In other experiments, whole, mature spinach plants were obtained from the store or from local farms. In the latter case, the cut plants were placed immediately in ice and used the same day. Unless specified only mature, undamaged leaves were used. All further steps in the preparation of the chloroplasts were carried out in the cold room at 4° C. The reagents and apparatus used in the procedure were also maintained at 4° C.

The method of preparing the chloroplasts is essentially that described by Arnon et al (1). The sliced leaves were ground in a mortar with 80 to 120 mesh silica (Amend Drug and Chemical Co., New York) and 0.35 M NaCl, adjusted to pH 7.5. Depending on the turgor of the leaves, approximately 1 ml of 0.35 M NaCl was used for each gram of blades. In later experiments, 20 micromoles (μM) of sodium ascorbate, pH 7.5, was added to the grinding mixture since it appeared to stabilize the CO_2-assimilating system. In order to facilitate the grinding and avoid foaming, leaves were ground in batches of 20 to 30 g. After the homogenate was gently squeezed through a double layer of cheesecloth, it was centrifuged at 200 × G for 1 minute (clinical model International) to sediment the larger fragments. The supernatant fluid was decanted and centrifuged at 1000 × G in the same machine 7 minutes. The sedimented whole chloroplasts together with some chloroplast fragments were suspended in 0.35 M NaCl (5 ml per 20 to 30 g leaf blades) by sucking them into a pipette tipped with cotton. This type of preparation is referred to as unwashed chloroplasts. The preparation obtained after a centrifugation of this suspension and another treatment with 0.35 M NaCl is referred to in this report as washed chloroplasts.

UPTAKE OF CARBON DIOXIDE: The fixation of isotope was carried out in 150 ml Warburg vessels whose main compartments contained the following components in μM: TRIS[3] buffer, pH 7.5, 80; sodium ascorbate, pH 7.5, 20; manganese chloride, 2; unlabelled $NaHCO_3$ 10; NaCl, 700. The total volume was 4.8 ml. The side arm carried 1 μM of $C^{14}O_2$ as $NaHCO_3$ containing 5.5×10^6 counts per minute in 0.2 ml 0.35 M NaCl. The adding of the reagents to the Warburg vessels was carried out in the cold room. After the vessels were flushed with N_2 in a bath at 14 to 17° C for 10 minutes with the lights on (approximately 1000 ft-c), the $NaHC^{14}O_3$ was tipped in. The light source was a bank of 8 Sylvania 30-watt daylight lamps. After 15 or 30 minutes, the reaction was terminated by adding 1 ml of 1 *N* HCl. For measurement of the total tracer fixed, 50 or 100 lambda duplicate aliquots of the vessel contents were pipetted onto aluminum planchets and evaporated to dryness under an infrared lamp. The dry samples were assayed with a gas-flow thin window counter.

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[3] The following abbreviations are used in this article: TRIS for Tris (hydroxymethyl)-aminomethane; TPN for triphosphopyridine nucleotide; PNH for reduced pyridine nucleotide; AMP, ADP and ATP for adenosine monophosphate, adenosine diphosphate and adenosine triphosphate; GSH for glutathione; p-CMB for para-chloromercuribenzoate; EDTA for ethylenediamine tetraacetic acid.

The chlorophyll content of each preparation was determined by the method of MacKinney (16) and, unless otherwise noted, aliquots containing 1 mg chlorophyll were used in each reaction vessel. All data are therefore expressed on the basis of 1 mg chlorophyll.

ENZYME EXPERIMENTS: Once washed chloroplasts were suspended for 30 minutes at 4° C in water adjusted to pH 7.5. Subsequently, the mixture was centrifuged at 20,000 × G for 20 minutes at 4° C. The supernatant solution was used for assay. Glyceraldehyde-3-phosphate dehydrogenase activity was determined in Beckmann cuvettes (d = 1 cm) with a total volume of 3 ml, and readings were made against a blank containing all reactants except fructose-1,6-diphosphate. The reaction mixture contained 100 μM TRIS buffer, pH 8.5, 51 μM of sodium arsenate, 0.2 μM TPN, chloroplast enzyme, and excess crystalline rabbit muscle aldolase. The reaction was started by adding 1.5 μM of fructose-1,6-diphosphate. Glucose-6-phosphate dehydrogenase assay was run in the same type of cuvette with a total volume of 3 ml and readings were made against a blank containing all reactants except glucose-6-phosphate. The reaction mixture contained 100 μM TRIS buffer, pH 8.5, 0.2 μM of TPN, 5 μM of magnesium chloride and chloroplast enzyme. The reaction was started by adding 5 μM of glucose-6-phosphate.

RESULTS

EFFECT OF LIGHT, NaCl WASHING AND SOURCE: The data in table I demonstrate the rapid acceleration of isotope incorporation due to the light. One 0.35 M NaCl washing reduced the light accelerated rate by half, while hardly affecting the dark rate. On the other hand, photophosphorylation activity is affected only slightly by one 0.35 M NaCl wash (6).

The rates recorded in this table are typical for leaves taken from plants grown in the hydroponic tank, however, on one occasion, we have observed 4.5 μM CO_2 fixed per hour per mg of chlorophyll. On the same basis, other materials gave values of: 0.6 (store bought, January and February), 0.9 (potted plant grown in same room as hydroponic material) and 0.8 (field grown, collected in August). Very little difference was found between the small, immature and fully expanded leaves.

TABLE I

EFFECT OF LIGHT AND 0.35 M NaCl WASHING OF CHLOROPLASTS ON CO_2 FIXATION

CONDITION	TOTAL CPM FIXED PER HR PER MG CHLOROPHYLL	μM CO_2 FIXED PER HR PER MG CHLOROPHYLL
Light—unwashed	1.4×10^5	2.5
Light—washed	7.2×10^5	1.3
Dark—unwashed	2.5×10^4	0.03
Dark—washed	2.1×10^4	0.03

TABLE II

EFFECT OF pH ON $C^{14}O_2$ FIXATION

TRIS		GLYCYLGLYCINE	
pH	$C^{14}O_2$ FIXED CPM/ALIQUOT	pH	$C^{14}O_2$ FIXED CPM/ALIQUOT
8.15	254	8.00	625
7.78	1030	7.68	1150
7.58	2960	7.51	2040
7.33	2480	7.20	1490

pH DEPENDENCE: A pH curve for chloroplast CO_2-fixation has not yet been published. The isotope incorporation rate proceeds at maximal velocity in the vicinity of pH 7.5 to 7.6 (table II). Below this pH, the fixation rate falls off slowly. Above this value, the rate declines sharply.

ACTION OF IODOACETAMIDE AND p-CMB: From the results outlined in table III, it is evident that the alkylating agent, iodoacetamide, and the mercaptide-forming compound, p-CMB, are both potent inhibitors of CO_2 fixation. It will be noted that a slightly higher concentration of inhibitor was required in experiments 2 and 4 in contrast to 1 and 3 suggesting that the unwashed chloroplast preparations contained endogenous sulfhydryl substances which were removed by the subsequent washing. One reason for the low incorporation of CO_2 by the chloroplasts may be the presence of heavy metals which are known to be inhibitors of sulfhydryl enzymes. Figure 1 illustrates the slow rate of TPN reduction with triose phosphate derived from fructose-1,6-diphosphate catalyzed by chloroplast glyceraldehyde-3-phosphate dehydrogenase. On the addition of EDTA, the rate of reduction rapidly increases apparently due to the chelating of heavy metals (curve 2). Curve 1 represents glucose-6-phosphate dehydrogenase activity and is used as a control on the possible conversion of fructose-1,6-diphosphate to glucose-6-phosphate. Since the rate of TPN reduction with glucose-6-phosphate is low, the assumption is made that curve 2 represents mainly glyceraldehyde-3-phosphate dehydrogenase. These data suggest that heavy metals may be a cause of lower fixation rates with store bought plants when contrasted to hydroponic material.

These results are qualitatively in agreement with those of Arnon et al (4) but on a quantitative basis the chloroplasts used in this study were somewhat more sensitive to the 2 sulfhydryl inhibition compounds.

ACTION OF p-CMB AND ARSENITE AND REVERSAL BY GSH: The inhibition by the mercuribenzoate can be prevented as well as reversed by GSH (table IV). In contrast to its complete protective action with respect to the mercaptide-forming agent, GSH provides the system with only a partial protection toward arsenite. This is indicative of a somewhat different mode of action of the 2 inhibitors and is in accord with the present concept of the action of a mercurial type inhibition which combines with thiols forming

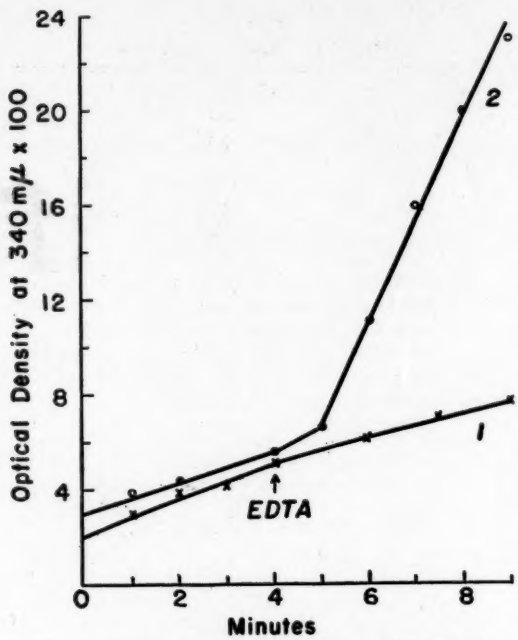

FIG. 1. Effect of ethylenediamine tetraacetic acid on the glyceraldehyde-3-phosphate (curve 2) and glucose-6-phosphate (curve 1) dehydrogenase of the spinach chloroplast. At arrow, 0.01 μM of EDTA added to each cuvette.

mercaptides which are easily reversed under the influence of another thiol of greater affinity (GSH) in contrast to metalloids like trivalent arsenic which form with thiol groups easily decomposed thioarsenites (7).

The action of GSH itself on the CO_2-fixing system should be mentioned because of its extreme variability. In 5 separate preparations, a molar concentration of 10^{-3} yielded the following values in percent of control fixation rate: +6, +40, −40, +29, −12. That GSH was inhibitory or had little effect was noted previously by Arnon et al (4). They did not report an increased rate due to the sulfhydryl compound. It may well be that in these experiments this compound plays both a protective role which would increase the rate as well as an electron transferring substance causing a draining off of reducing potential resulting in a lower fixation of CO_2.

ACTION OF ARSENATE AND INORGANIC PHOSPHATE: At low concentrations, both arsenate (table V) and phosphate (table VI) stimulate the rate, however, a diminution occurs as the concentration of the 2 ions is increased. These data would suggest that the 2 compounds play a similar role in the overall process measured by the incorporation of CO_2. On the basis of inorganic phosphate in higher concentration having an inhibitory effect on CO_2 fixation, the speculation (1) was made that a competitive relation exists between the photophosphorylation and CO_2-fixation systems of the chloroplast. Our experiments do not deny this view but they do provide evidence that the effect of phosphate may have another explanation.

The data of table VII illustrate that the inhibition caused by arsenate is not competitive with phosphate. This would again suggest that photosynthetic phosphorylation is not primarily involved since Avron and Jagendorf (5) have found that arsenate is an inhibitor of ATP formation, competitive with phosphate.

A possible explanation for the effect of phosphate and arsenate may be found in their action toward individual enzymes of the carbon cycle. The enzyme catalyzing the carboxylation of ribulose diphosphate has been reported to be inhibited by arsenate and phosphate (24). In this latter work, much higher concentrations of the 2 ions were needed to give inhibitions with the purified enzyme comparable to those found with the chloroplast. Unfortunately, the activity of the carboxylating enzyme toward lower concentrations was not reported. The possibility exists that not only is the enzyme(s) responsible for carboxylation inhibited but that possibly the enzyme(s) catalyzing the reduction of the carboxyl group or other enzymes of the cycle sensitive to phosphate and arsenate.

TABLE III

EFFECT OF IODOACETAMIDE AND p-CMB ON $C^{14}O_2$ FIXATION

EXPERIMENT	INHIBITOR M CONC	$C^{14}O_2$ FIXED CPM/ALIQUOT	% INHIBITION*
	0	650	...
	5×10^{-4} Iodoacetamide	610	6
1	1×10^{-3} Iodoacetamide	600	8
	5×10^{-3} Iodoacetamide	250	62
	5×10^{-4} Iodoacetamide	7	99
	0	1630	...
2	2×10^{-4} Iodoacetamide	1160	29
	5×10^{-4} Iodoacetamide	315	80
	0	1690	...
	5×10^{-4} p-CMB	1690	0
3	1×10^{-3} p-CMB	1460	14
	5×10^{-3} p-CMB	205	88
	1×10^{-4} p-CMB	71	96
4	0	317	...
	5×10^{-3} p-CMB	189	40

Washed chloroplasts were used in experiments 1 and 3; unwashed chloroplasts in experiments 2 and 4. The inhibitors were incubated with the reaction mixture 20 minutes in the dark at 4° C; then an additional 10 minutes in the Warburg bath during the nitrogen gas flushing with the lights on before the $NaHC^{14}O_3$ was tipped in. The $C^{14}O_2$ fixed figures have been corrected for dark fixation. This dark value for each experiment is: (1) 35, (2) 25, (3) 33, (4) 23. Dark fixation was affected slightly by the inhibitors.

* Percent inhibition = (cpm (control) − cpm (inhibited))/cpm (control) × 100.

The organization of the enzymes within the chloroplast must surely have a quantitative effect on such studies.

EFFECT OF AMMONIA SALTS: An uncoupling of photosynthetic phosphorylation by ammonium ion has been reported (15). Since none of the enzymes of the photosynthetic carbon cycle are known to be inhibited by this ion, inhibition of CO_2-fixation, relieved by ATP would suggest a coupling of photophosphorylation to CO_2-reduction. While NH_4^+ strongly inhibited CO_2-fixation (table VIII), the addition of ATP had little effect. The inaccessibility of the active sites to ATP may be given as an explanation, however, these preparations will carry out photophosphorylation with AMP and ADP as acceptor.

DISCUSSION

Photosynthesis is considered to consist of a light and dark phase. The photochemical portion is characterized by the photolysis of water and is generally thought to end with the formation of the 2 chemical materials, PNH and ATP, needed to assimilate CO_2 to the carbohydrate level. The basis for assigning this role to these compounds is due to many factors among which are: 1) that the reduction in photosynthesis involves the conversion of CO_2 or carboxyl to the aldehyde level and by analogy to known enzymatically catalyzed reductions involving carboxyl carbons, occurs in 2 steps involving ATP and PNH and 2) that the enzyme systems catalyzing pyridine nucleotide reduction and photophosphorylation have been detected thus far only in chloroplasts and chromatophores which are the particles of autotrophic cells concerned with photosynthetic carbon assimilation. Others (10) have speculated that the reductant is a single compound with a sufficiently low reducing potential capable of catalyzing the reaction in a single step. A compound of this type is implied in the hypothesis advanced by Arnon et al (4) that photosynthetic phosphorylation by isolated chloroplasts is coupled to pyridine nucleotide reduction. It is possible that under the experimental conditions employed with isolated chloroplasts in which stoichiometry is shown between O_2 evolution, pyridine nucleotide reduction and esterification of orthophosphate in the absence of carbon assimilation, there may be obtained a misleading expression of the reducing agent (H) derived from water since its natural reactant, CO_2, is absent. Thus far there is no evidence for a photosynthetic reductant other than ATP and PNH.

Although there are dangers involved in extrapolation from the artificial system of the isolated chloroplast to the situation in the whole cell, still it is interesting to note that rates of photophosphorylation up to 700 μM of orthophosphate esterified per hour per mg of chlorophyll (2, 13) and rates of 75 μM of TPN reduced per hour per mg of chlorophyll (20) have been published. A comparison of these rates with the observed CO_2-fixation rate of 2 to 4 μM per hour per mg of chlorophyll presented here and by others (1)

TABLE V

EFFECT OF ARSENATE ON $C^{14}O_2$ FIXATION BY WASHED CHLOROPLASTS

EXPERIMENT	ARSENATE M CONC	$C^{14}O_2$ FIXED CPM/ALIQUOT
1	0	678
	1×10^{-6}	1580
	5×10^{-6}	588
	1×10^{-5}	498
	5×10^{-5}	109
	1×10^{-4}	71
2	0	422
	5×10^{-7}	469
	1×10^{-6}	857
	5×10^{-6}	1050
	1×10^{-5}	291

TABLE VI

EFFECT OF PHOSPHATE OF $C^{14}O_2$ FIXATION BY UNWASHED CHLOROPLASTS

EXPERIMENT	PHOSPHATE M CONC	$C^{14}O_2$ FIXED CPM/ALIQUOT
1	0	2370
	2×10^{-4}	3200
	1×10^{-3}	2570
	2×10^{-3}	1050
2	0	2130
	1×10^{-3}	3040
	5×10^{-3}	176
	1×10^{-2}	154

TABLE IV

REACTIVATION WITH GSH AFTER INHIBITION BY p-CMB AND ARSENITE

COMPOUNDS M CONC	$C^{14}O_2$ FIXED CPM/ALIQUOT	% INHIBITION+	% REACTIVATION‡
Complete	781	...	...
GSH 10^{-3}	700	...	...
GSH 10^{-3}, arsenite 10^{-4}*	361	...	22
Arsenite 10^{-4}, GSH 10^{-3}**	308	...	13
Arsenite 10^{-4}	251	68	...
Complete	317	...	...
GSH 10^{-3}	453	...	...
GSH 10^{-3}, p-CMB 5×10^{-5}*	440	...	95
p-CMB 5×10^{-5}, GSH 10^{-3}**	555	...	100
p-CMB 5×10^{-5}	189	40	...

Washed chloroplasts were used.

* Order of addition, GSH at zero time; inhibitor 10 minutes later.

** Order of addition, inhibitor at zero time; GSH 10 minutes later.

+ Percent inhibition = (cpm (control) − cpm (inhibitor))/cpm (control) × 100.

‡ Percent reactivation = (cpm (after reactivation) − cpm (inhibitor))/cpm (GSH) − cpm (inhibitor)) × 100.

TABLE VII

COMBINED EFFECT OF ARSENATE AND PHOSPHATE ON $C^{14}O_2$ FIXATION BY UNWASHED CHLOROPLASTS

ARSENATE M CONC	PHOSPHATE M CONC	$C^{14}O_2$ FIXED CPM/ALIQUOT
...	...	1000
...	2×10^{-4}	2550
2×10^{-4}	...	190
2×10^{-4}	2×10^{-4}	218
2×10^{-4}	6×10^{-4}	243

leads to the conclusion that while the capacity of chloroplasts to convert light energy into ATP and PNH is significant, the fixation of CO_2 is not accelerated in proportion. Possible causes may be leakage of the carbon cycle enzymes (18) and disruption of the organization of the chloroplast by isolation with 0.35 M NaCl. The finding of Thomas et al (22) that the rate of CO_2 reduction was of the same order of magnitude for suspensions of fragmented Spirogyra chloroplasts as those observed in whole cells led them to the conclusion that the stroma of lamellated chloroplasts remain intact during isolation in contrast to that of spinach chloroplasts. One difference between the data obtained with Spirogyra fragments and that observed with spinach chloroplasts is the lack of inhibition of algal CO_2-fixation with 0.2 M phosphate.

Even though we are measuring the dependence of pH on the total reaction rather than on an isolated portion of the reaction, enough information has been published on some of the individual steps to warrant comment. The pH optimum for the spinach CO_2-fixing system contrasts sharply with the pH of maximal rate for photophosphorylation. The spinach phosphorylation system with AMP as acceptor has a broad pH range peaking between 7.8 to 8.0 (8, 9) while the reaction with ADP has been reported to have a sharp pH optimum at 8.3 (8). A preliminary note of Walker and Hill (23) indicates that photophosphorylation obtained with pyocyanine and ADP showed 2 maxima, a higher one at pH 7 and a shoulder at pH 7.7. With aged chloroplasts, they found only a single maximum at pH 7.7. Based on the CO_2-activity curve, it would appear that the labile phosphorylation system detected by Walker and Hill may play a more important role in the overall photosynthetic process than the more stable one at the higher pH optimum. Similarly photosynthetic pyridine nucleotide reductase (20) shows a pH optimum at 6.9 and the fructose-1,6-diphosphatase of spinach chloroplasts has its maximum rate at 6.9 in contrast to the spinach cytoplasm fructose-1,6-diphosphatase which acts optimally at pH 8.5 (19).

TABLE VIII

EFFECT OF AMMONIUM ION ON $C^{14}O_2$ FIXATION BY UNWASHED CHLOROPLASTS

NH^+_4 M CONC	$C^{14}O_2$ FIXED CPM/ALIQUOT	% INHIBITION*
0	2800	...
1×10^{-3}	525	81
2×10^{-3}	300	89
1×10^{-2}	30	99
2×10^{-3} plus 3×10^{-4} ATP	303	89

* Percent inhibition = (cpm (control) − cpm (inhibitor))/cpm (control) × 100.

Data on the effect of sulfhydryl inhibitors on various reactions of the photosynthetic process have been recorded by San Pietro and Lang (20), Arnon et al (4) and in this paper. The 2 which warrant discussion are iodoacetamide and arsenite since both inhibit CO_2 fixation ((4), table III) but have no effect on the photolysis reaction (4), photosynthetic phosphorylation (4) and the photosynthetic pyridine nucleotide reductase (20). The effect of the alkylating agent may be due to inhibition of glyceraldehyde-3-phosphate dehydrogenase, however, ribose-5-phosphate isomerase (21) and phosphoribulokinase (18) which are generally considered to be involved in photosynthetic carbon assimilation are sulfhydryl enzymes but their sensitivity toward this inhibitor has not been tested. The site of arsenite inhibition is puzzling. Arsenite inhibits the overall chloroplast system when assayed by the uptake of $C^{14}O_2$ ((4), table IV), however, none of the reactions of the photochemical act or of the dark phase of photosynthesis tested thus far are affected. Inhibition by trivalent arsenic is under investigation in this laboratory and will be the subject of a forthcoming paper.

SUMMARY

The effect of various factors on the light induced fixation of $C^{14}O_2$ by isolated spinach chloroplasts has been investigated. The rate routinely observed with hydroponic grown material was 2.5 μM of CO_2 assimilated per hour per mg chlorophyll. Lower rates were obtained with field grown, store bought or potted plants. The pH optimum of the system was 7.5 to 7.6. Observation of the action of several metabolic inhibitors revealed that iodoacetamide, p-chloromercuribenzoate and arsenite were highly effective in inhibiting $C^{14}O_2$-fixation. Arsenite inhibition, in contrast to the inhibition effect by p-chloromercuribenzoate, could not be prevented markedly by glutathione. This would suggest a specific effect of the trivalent arsenical. While low concentration of arsenate (10^{-4}) and phosphate (10^{-4}) accelerated carbon assimilation, higher concentration of each caused strong inhibition. The inhibition effected by the ammonium ion was not relieved by adenosine triphosphate. The significance of these findings are evaluated in relation to other light induced reactions of the chloroplast.

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INTRACELLULAR AND PHYLOGENETIC DISTRIBUTION OF RIBULOSE 1,5-DIPHOSPHATE CARBOXYLASE AND D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES [1, 2]

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The main reaction through which CO_2 enters into photosynthesis has been established to be the carboxylation of RuDP[3] to form 2 molecules of PGA. This reaction has been found to occur in intact algae (2) and in cell-free preparations of several organisms (11, 12, 16). The reaction occurs with a large loss in free energy (2, 16), and in this aspect differs from most biological carboxylations. Under certain conditions, this property would have a selective advantage as a reaction to be retained where any type of CO_2 fixation were occurring.

Speculation has been raised that a TPN requiring G-3-P dehydrogenase is also closely connected with photosynthesis. Gibbs (7) reported that whereas pea root extracts contained a DPN-linked dehydrogenase similar to that found in heterotrophic tissues, extracts of pea leaves possessed not only the DPN enzyme but also a TPN requiring enzyme. This observation was confirmed by Rosenberg and Arnon (13). A relationship between the two dehydrogenases during the life cycle of the pea plant and the effect of light on this relationship has been reported by Hageman and Arnon (8). It is interesting to note that Trudinger (15) could demonstrate a RuDP carboxylating enzyme but only a DPN-linked triose phosphate dehydrogenase in extracts of *Thiobacillus denitrificans*.

It would be of interest, therefore, from an evolutionary viewpoint to determine if these enzymes function merely in autotrophic metabolism or whether they might also play a role in other metabolic pathways. The purpose of this report is to present evidence that the RuDP carboxylase and the TPN-linked G-3-P dehydrogenase are not found exclusively in autotrophic metabolism. Data are also given which demonstrate an association between the carboxylating enzyme and the chloroplast. In addition to working with isolated fragments of the cell which are always of questionable purity it was felt that perhaps a less direct but more realistic physiological approach would be to investigate actively metabolizing cells in which the chloroplast had been altered or removed by external variables. Two preliminary reports of this investigation have been published (4, 5).

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[2] Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission and in part supported by a grant from the National Science Foundation to one of us (M.G.).

[3] Abbreviations used in this paper: PGA for 3-phosphoglyceric acid; TPN for triphosphopyridine nucleotide; DPN for diphosphopyridine nucleotide; G-3-P for D-glyceraldehyde-3-phosphate; RuDP for ribulose 1,5-diphosphate.

MATERIALS AND METHODS

All the algae used in this work were grown on a continuous culture, constant temperature, illuminated shaker with approximately 2,000 ft-c illumination during the growth cycle. Cells were harvested every 24 hours and the amount of cellular material determined by both dry weight and amount of chlorophyll per ml of culture. When bleached cells and Astasia were grown, 0.5 % sodium acetate was added to the medium as the sole source of carbon. The Chromatium, strain D, was grown as an autotroph according to the method of Hendley (9). Heterotrophic Chromatium, strain D, was grown by adding 0.5 % sodium acetate to the medium. The medium for the glucose grown *Escherichia coli* strain Crookes, is that of Gest and Gibbs (6). The D-xylose adapted *E. coli* cells were grown on a mineral medium containing 0.5 % D-xylose with a 5 % CO_2 - 95 % air mixture being bubbled at a rapid rate through the medium. All higher plants were from stock greenhouse material.

The cellular material to be assayed for enzyme activity was prepared in 2 ways. The algae and bacteria were harvested and made into a 20 % wet weight suspension in 0.01 M K-phosphate buffer at pH 6.5 and then treated in a 10 Kc Raytheon oscillator for 15 minutes. The residual fragmented material was centrifuged at 1,000 × G for 15 minutes and the supernatant liquid was tested immediately for enzyme activity. Equal amounts of green and albino barley were ground with sand and 0.3 % ethylenediamine tetraacetate at pH 8.0 in 0.5 M $KHCO_3$. The soluble material was separated by centrifugation at 18,000 × G for 10 minutes. Spinach chloroplasts were prepared by the method of Arnon et al (1).

Assay for RuDP carboxylase activity was made in the following way. The material to be assayed was incubated for 1 minute with 0.1 micromoles (μM) of $NaHC^{14}O_3$ containing 10 microcuries and with 0.1

μM of RuDP. The Ba salt of RuDP was converted to the Na salt by treatment with Dowex-50 (Na^+). The subsequent formation of labeled PGA, which was observed and identified by paper chromatography and radioautography, was taken to indicate the presence of the enzyme. In each assay, a control was run in the absence of RuDP and in each case PGA was not detected (fig 1).

The chromatograms were first developed in phenol : water followed by treatment with butanol : propionic acid : water as described by Benson et al (3). All radioactive products were counted directly on the chromatograms and expressed as counts per minute.

G-3-P dehydrogenase activity was determined by the following procedure. The assays were run in Beckmann cuvettes (d = 1 cm) with a total volume of 3 ml and readings were made against a blank containing all reactants except pyridine nucleotide. The reaction mixture which contained 100 μM of tris (hydroxymethyl)-aminomethane, pH 8.0; 17 μM of sodium arsenate; 12 μM of cysteine, pH 8.0; 0.18 μM of pyridine nucleotide and enzyme were incubated 7 minutes before the addition of 0.5 μM of G-3-P. Readings were begun 30 seconds after the addition of the triose phosphate. The DL-G-3-P was prepared from the dioxane addition compound of DL-glyceraldehyde-1-bromide-3-phosphoric acid (dimer).

Results and Discussion

Enzyme Activity: A series of albino mutants prepared by x-irradiation of barley seed were made available through the kindness of Dr. Calvin Konzak of this laboratory. Week-old normal and mutant seedlings were used to prepare extracts for the assay of RuDP carboxylase and the G-3-P dehydrogenases. The results of these experiments are shown in table I. The normal green seedling contains a highly active carboxylating system. In the control assay small amounts of radioactivity are fixed and most isotope was located in malic acid. With the addition of RuDP there is a large increase in the total fixation, almost all of which is accounted for by formation of PGA. There was a small increase also in the formation of

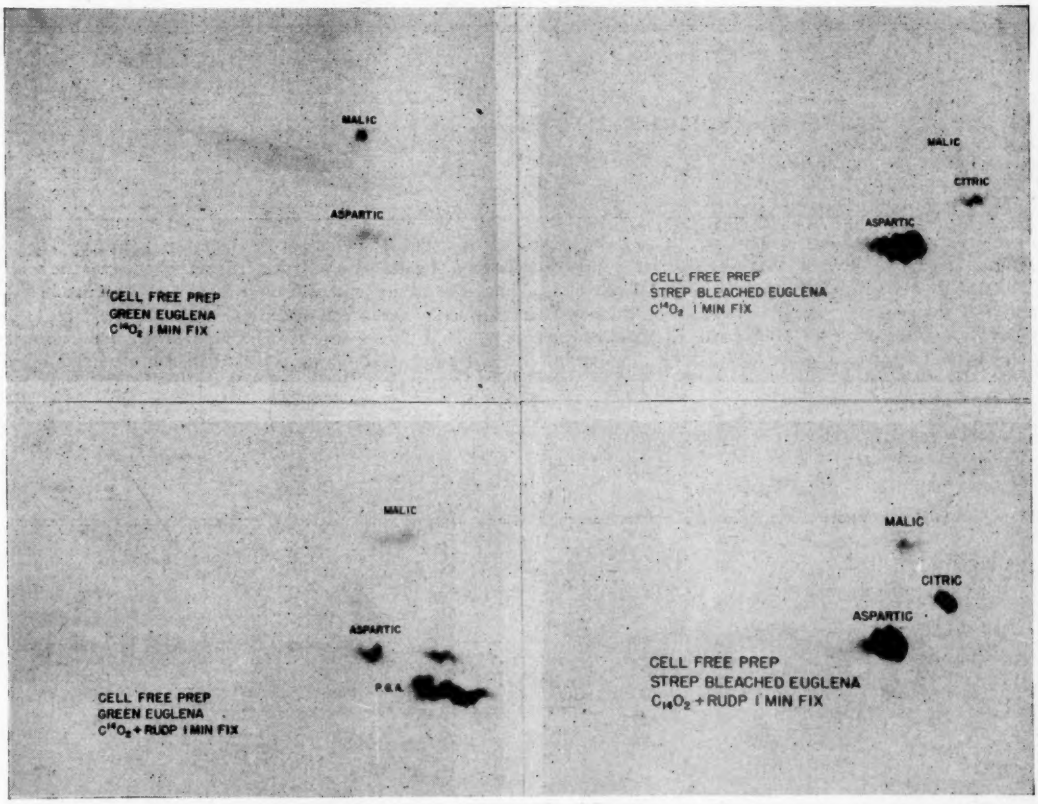

Fig. 1 (*left, top and bottom*). Ribulose diphosphate carboxylase in green Euglena.

Fig. 2 (*right, top and bottom*). The effect of streptomycin on the ribulose diphosphate carboxylase of Euglena. (Increase in $C^{14}O_2$ fixation into aspartic acid is probably due to the increased oxidative metabolism of an acetate grown organism.)

malic acid and a few other products. The same assay was then carried out on the x-ray induced albino mutants. Here, again, in the control experiment there is a small but significant fixation of isotope in the malic acid. Upon the addition of RuDP there is no increase in the amount of total fixation by the extract and there is no formation of PGA. It, therefore, appears that these albino mutants have lost the ability to carboxylate RuDP. In contrast to the RuDP carboxylase, both the normal and the mutant barley extracts possess both dehydrogenases (table I). This suggests that the TPN-requiring enzyme may develop independently of the photosynthetic process. It is interesting to note that whereas the activities of the G-3-P dehydrogenases are essentially equal when fresh weight is used as a basis for comparison, the albino values show a 6-fold higher specific activity on a protein basis. The status of the chloroplast in these cells is not clear. There is no chlorophyll present, since neither the a nor b peak could be observed spectrophotometrically in acetone extracts. These albino plants contain 10 times more carotenoids than the wild type green plant.

A different situation is demonstrated in table II where data are presented for a series of Euglenoids. Here it is possible to obtain from a single form, organisms in which the chloroplast of the cell is altered in several ways. The initial form is the normal green flagellate. It is possible to grow the organisms in the dark for a period of several weeks whereupon they become completely colorless and do not contain any detectable chlorophyll. However, upon re-exposure to light these organisms rapidly form visible chloroplasts and chlorophyll. It was demonstrated a number of years ago by Provasoli et al (10) that streptomycin bleached irreversably the chloroplasts of Euglena. Normal green cells were treated in this manner and the resulting colorless forms were maintained as heterotrophs on acetate as a sole source of carbon. There is available the natural colorless vari-

TABLE I

ENZYME ACTIVITY IN BARLEY SEEDLINGS

TISSUE FROM WHICH EXTRACTS WERE PREPARED	RuDP CARBOXYLASE			G-3-P DEHYDROGENASE*	
	$C^{14}O_2$ FIXED 1 MIN	PGA	MALIC ACID	TPN	DPN
	CPM	CPM	CPM		
Normal green seedling	450	0**	275	10	0.2
Normal green seedling + RuDP	7200	5180	710	...	...
x-ray induced albino mutant	200	0	200	1.8	0.03
x-ray induced albino mutant + RuDP	350	0	300	...	...

* The determination of G-3-P dehydrogenase was done in the following way. About 35 five-day-old plants (albino—1.9 g fresh weight; green—2.1 g fresh weight) were ground with 100 μM of ethylene diamine tetraacetic acid, pH 8.0, 500 μM of $KHCO_3$ and sand in a total volume of 3 ml. The homogenate was centrifuged for 10 min at 18,000 $\times$ G. The supernatant fluid was used directly for protein analysis by the trichloroacetic acid method (14) and for G-3-P dehydrogenase (see Methods section). Protein content of the albino extract was 1.8 mg per ml while normal green extracts showed a protein value of 12.2 mg per ml. Enzyme activity is given as the change in optical density $\times 10^3$ the 1st 5 minutes after the addition of DL-G-3-P per mg of protein.

** 0 signifies no detectable activity on x-ray film.

TABLE II

ENZYME ACTIVITY IN EUGLENOIDS

TISSUE FROM WHICH EXTRACTS WERE PREPARED	RuDP CARBOXYLASE				G-3-P DEHYDROGENASE	
	$C^{14}O_2$ FIXED 1 MIN PER 10 MG CELLS	PGA	ASPARTIC ACID	MALIC ACID	TPN	DPN
	CPM	CPM	CPM	CPM		
Green Euglena	400	100	110	180	14**	47
Green Euglena + RuDP	1400	1350	110	120	...	...
Bleached Euglena	1200	0*	1200	20	0	40
Bleached Euglena + RuDP	1200	0	1100	40	...	...
Dark grown Euglena	1100	0	1000	20	0	50
Dark grown Euglena + RuDP***	800	170	60	20	...	...
Astasia	200	0	100	50	0	46
Astasia + RuDP***	1100	450	100	75	...	...

* 0 signifies no detectable activity on x-ray film.

** Change in optical density $\times 10^3$ the 1st 5 minutes after the additon of DL-G-3-P.

*** The apparent loss of total activity in these 2 cases is accounted for by the formation of fatty acids and lipid. They appear on the chromatogram but were not counted as the compounds were not identified.

ant of these forms, Astasia, which was also tested.

The data presented in table II show clearly that there is an active RuDP carboxylase in the extracts of the green Euglena. Considerable amounts of both aspartic and malic acids are also formed in the control fixations with these cells (fig 1). Extracts of the streptomycin bleached cells fixed large amounts of CO_2 in aspartic acid and some in malic acid but neither in the absence nor presence of RuDP was PGA formed (fig 2). The dark-grown Euglena extracts do fix a slight amount of activity. The data recorded in table II also show that of all the Euglenoids tested, only the chloroplast-containing cells possessed both TPN and DPN-linked G-3-P dehydrogenases. In this table, specific activity of the dehydrogenases is not based on protein concentration but on equal amounts of fresh weight of algae. On this basis the various altered forms apparently lose the TPN-requiring system, while the amount of DPN activity is not significantly altered.

It is evidently extremely easy with these microorganisms to remove the carboxylating enzyme and the TPN-dehydrogenase by either removing the chloroplast with streptomycin or diminishing photosynthetic activity by bleaching the cells in the dark. However, Astasia, which has presumably arisen evolutionarily from Euglena, has apparently retained the carboxylating enzyme. It may play an important role in pentose metabolism in this form. Carbon dioxide stimulates the growth of this organism and perhaps this is the mechanism for such a stimulation.

Chlorella variegata, which is normally an autotroph but which loses the major portion of its chlorophyll when grown on an organic source of carbon in the light, was also used as a test organism. Two cultures

TABLE III

ENZYME ACTIVITY IN CHLORELLA VARIEGATA

EXTRACTS FROM CELLS GROWN AS	RuDP CARBOXYLASE			G-3-P DEHYDROGENASE	
	$C^{14}O_2$ FIXED IN 1 MIN PER 10 MG CELLS	PGA	MALIC ACID	TPN	DPN
	CPM	CPM	CPM		
Autotrophs	400	0*	250	...	...
Autotrophs + RuDP	3050	2550	300	...	...
Heterotrophs	400	0	320	0**	50
Heterotrophs + RuDP	425	0	360	...	...

* 0 signifies no detectable activity on x-ray film.
** Change in optical density $\times 10^3$ the 1st 5 minutes after the addition of DL-G-3-P.

TABLE IV

OCCURRENCE OF ENZYMES IN VARIOUS MICROORGANISMS

ORGANISM*		RuDP CARBOXYLASE		G-3-P DEHYDROGENASE**	
		CPM TOTAL FIXATION	CPM PGA	TPN	DPN
Tetrahymena gelii	− RuDP	1,650	0	0	11
	+ RuDP	1,500	0		
Yeast	− RuDP	1,300	0	...	...
	+ RuDP	1,225	0		
Neurospora crassa	− RuDP	920	0	...	...
	+ RuDP	865	0		
Rhodospirillum rubrum	− RuDP	1,100	0	10	30
	+ RuDP	22,550	9,885		
Anacystis nidulans	− RuDP	350	0	12	2
	+ RuDP	40,275	18,500		
Chromatium, strain D autotrophic	− RuDP	0	0	...	...
	+ RuDP	56,065	12,500		
heterotrophic	− RuDP	500	0	...	...
	+ RuDP	41,500	10,900		
Escherichia coli (Crookes) grown on glucose	− RuDP	8,300	0	...	...
	+ RuDP	7,700	0		
grown on xylose + CO_2	− RuDP	10,400	0	...	...
	+ RuDP	40,200	2,750		

* Where a comparison of autotrophic and heterotrophic forms is used, equal weights of cellular material are used.
** Enzyme activity is given as the change in optical density $\times 10^3$ the 1st 5 minutes after the addition of DL-G-3-P.

of this organism were grown in the light under identical conditions with the exception that in one case sodium acetate was added to the medium. In the heterotrophic culture, after several generations, most of the chlorophyll disappeared. Samples of both cultures were then tested for the presence of the carboxylating system. The results shown on table III indicate that in the light when this organism is metabolizing as a heterotroph it does not maintain the ability to carboxylate RuDP. These results are in agreement with those obtained with *Hydrogenomonas vinelandii* which when grown as an autotroph can carboxylate RuDP and cannot when metabolizing heterotrophically (Vishniac, personal communication). It therefore seems apparent that a variety of external variables can effect the functioning of the carboxylating system. Certainly in removing the chloroplast and consequently destroying the photochemical apparatus the carboxylating system becomes inactive. Apparently by allowing *C. variegata* and *H. vinelandii* to use a reduced source of carbon for energy the autotrophic carbon assimilation mechanism becomes inactive. In regard to G-3-P dehydrogenase activity, our results recorded in table III show that the TPN enzyme is absent in heterotrophically grown *C. variegata*.

Table IV summarizes our finding with 4 non-photosynthetic organisms, *Tetrahymena geleii*, yeast, *Neurospora crassa* and *Escherichia coli* and 3 organisms, *Rhodospirillum rubrum*, *Anacystis nidulans* and Chromatium, strain D, normally considered to be autotrophic.

The triose phosphate dehydrogenase data are consistent with our other findings, but 2 exceptions will be noted in the RuDP carboxylase column.

E. coli when grown on D-xylose and 5 % CO_2 is one exception. Extracts of this organism possess the carboxylating enzyme. We have taken this as convincing evidence indicating that this enzyme is not the exclusive property of the autotrophic organisms. In contrast to several organisms described Chromatium, strain D, can carboxylate RuDP when grown either as an autotroph or heterotroph.

RuDP Carboxylase Distribution: The distribution of this enzyme in various fractions of the spinach plant are recorded in table V. It would appear that it is possible to separate the highly organized chloroplast of spinach from the rest of the cellular material in such a way as to have most of the RuDP carboxylase associated with the chloroplast. One washing with water, which disorganizes the chloroplast, produces most of the enzyme in a soluble form free of the remaining chlorophyll-containing particles.

Summary

A survey has been made for the presence of the ribulose diphosphate carboxylase and the triphosphopyridine nucleotide and diphosphopyridine nucleotide dependent D-glyceraldehyde-3-phosphate dehydrogenase in some autotrophic and heterotrophic organisms. In general, the autotrophs contain the 3 enzymes while the heterotrophs lack the triphosphopyridine nucleotide linked dehydrogenase. The exceptions are Astasia, a naturally colorless Euglena, and D-xylose plus CO_2 grown *Escherichia coli* which contain the carboxylase and a genetic albino barley mutant which did possess the triphosphopyridine nucleotide dependent dehydrogenase. On fractionation of spinach leaves, most of the carboxylase activation was found to be associated with the chloroplast.

The authors wish to express their appreciation to Professor E. Baer for the dioxane compound of glyceraldehyde-1-bromide-3-phosphoric acid and to Dr. B. L. Horecker for the barium salt of ribulose diphosphate. They also wish to express their thanks to Mr. Frank German for his continuing help in providing plants for these investigations.

Table V

Distribution of RuDP Carboxylase in Fractions of Spinach Leaf

Plant fraction*	Phosphoglyceric acid		Aspartic acid	Malic acid	Glutamic acid
	$C^{14}O_2$** no RuDP	$C^{14}O_2$ RuDP			
I. Supernatant from ground leaves (plastid free)	...	3000	2000	700	...
II. Low speed fraction (chloroplasts)	0***	9000	...	400	...
III. High speed fraction (broken chloroplasts and other particles)	...	500	200	50	100
IV. H_2O extract of chloroplasts from I	...	8000	...	200	...
V. Residue from H_2O extract	...	1500	...	...	...

* All fractions were diluted for use in the assay so that equivalent amounts of plant material based on a chlorophyll or wet weight basis were used. Therefore, counts of PGA give a direct indication of distribution of enzyme.

** Data in cpm; $C^{14}O_2$ fixed in a 10 minute assay.

*** 0 signifies no detectable activity on x-ray film.

Part of these data appeared in the Quarterly Progress Report of the University of California Radiation Laboratory, 1955 (UCRL no. 1932.).

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BIOSYNTHESIS AND POSSIBLE FUNCTIONAL RELATIONSHIPS AMONG THE CAROTENOIDS; AND BETWEEN CHLOROPHYLL A AND CHLOROPHYLL B [1, 2]

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It is generally conceded that the carotenoids must have some indispensable function in photosynthesis. This has been variously formulated in terms of energy transport to and from chlorophyll (2, 3, 12), energy conversion and, or electron transport (4, 11) and O_2 transport (8). These may all be manifestations of the same act. In addition to the above types of functions, a photo-oxidation protection mechanism has been suggested (9) and more recently a non-photosynthetic O_2 transport function (O_2 absorption) also has been added (6).

An early attempt to demonstrate the photosynthetic O_2 transport function, using H_2O^{18} was unsuccessful (6). In preparing for a second such attempt, an experiment in which the carotenoids were labeled with C^{14} was devised.

During the course of the present work there appeared a publication (13) which indicated a reciprocal relationship between xanthophyll and a diepoxide related to it, violaxanthin, which was influenced by light; in the dark the xanthophyll concentration decreases and that of the violaxanthin increases, while the reverse reaction is induced by the light. This would have as a consequence a requirement that the specific radioactivity of C^{14}-labeled violaxanthin and xanthophyll should be very nearly equal. A similar specific activity relationship between chlorophyll a and chlorophyll b should be attained, if they were also involved in a rapid interconversion process. It is the purpose of the present investigation to determine the relative specific activities of a number of these pigments under labeling conditions using the algae, Chlorella and Scenedesmus.

Materials and Methods

The Algae used in these experiments were *Chlorella pyrenoidosa* Chick and *Scenedesmus obliquus* (Turpin) Kuetying, grown under our laboratory con-

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ditions (1 a). For this study, Scenedesmus (1.2 ml wet-packed cells in 60 ml nutrient solution) was allowed to photosynthesize for 1 hour with 0.5 ml (200 microcurie) of 0.036 M $NaHCO_3$. The cells were aerated with 4 % CO_2-in-air and illuminated with 3000 ft-c of light. At the end of the 1-hour photosynthesis period the algae were centrifuged and extracted twice with 10 ml boiling methanol (in those experiments comparing the pigment concentrations in light and dark the algae were killed in 0.02 M KCN).

In order to obtain quantitative figures concerning the radioactivity, improved techniques for pigment separation were needed, especially for the carotenoids with higher O_2 contents. Since it was necessary to obtain the actual pigment concentration in the plant, the method of separation should cause no alteration to the pigments, which are all rather unstable to light, O_2, acidity etc. This condition excluded a number of conventional separation methods like chromatography on alumina columns. An elution chromatography in polyethylene powder columns proved to be satisfactory for the various plant pigments.

The extract was chromatographed on a column of 15 g of polyethylene (re-used low density product of molecular weight about 360,000) which was added as a slurry in 80 % aqueous methanol. After packing the column under a pressure of about 0.05 atmos. it was washed with a solution of 1 g per liter EDTA-Na_4 (tetra sodium salt) in methanol : water 1 : 1. The chromatogram was developed with 80 % aqueous methanol and its colored zones were successively eluted. The resulting solutions of the pigments violaxanthin, C5, C4, xanthophyll-epoxide, C3, xanthophyll and a mixture of the carotenes and chlorophylls were concentrated in vacuo to 20 to 40 ml, diluted with 5 % aqueous sodium chloride solution and extracted with 2 to 10 ml benzene (according to the pigment concentration). The benzene extracts were kept frozen until use.

The above described method of polyethylene chromatography was sufficient for spectroscopic determinations of the pigment concentrations, but it did not allow the pigments to be purified enough to determine their radioactivity. The pigment elution fractions were always accompanied by colorless radioactive substances, which could be easily shown by paper chromatography. It was therefore necessary to apply a 2nd separation to the fractions from polyethylene chromatograms. This could be achieved for most of the pigments by two-dimensional paper chromatography.

Aliquot portions of these pigment solutions in benzene were applied to Whatman no. 3 MM paper. Ascending chromatograms were made in the dark using toluene as 1st, and petroleum ether (b.p. 65 to 110° C) + 2 % *n*-butanol as 2nd solvent for the carotenoids (3 % instead of 2 % *n*-butanol for the green pigments). The radioactive areas on paper chromatograms were detected by exposing the paper to DuPont x-ray film type 507.

Since the following determination of the molar radioactivity of the pigments had to be done by measuring the light absorption and radioactivity in the same sample, the pigment was required in solution. Hence the difficulty arose of elution of the colored spots from paper chromatograms without alteration of the pigments. A number of solvent systems were investigated in this respect; a mixture of methanol + benzene 4 : 1 was used for the carotenoids, and isopropylether + isopropanol 9 : 1 for the chlorophylls. The pigment spots were cut out and eluted with the above ascending solvents into small beakers. When the pigment accumulated in the upper end of the paper, this end was cut out and extracted in a test tube (the methanol solvent had to be heated).

In the case of the carotenes, however, calcium hydroxide columns were used as the 2nd chromatography medium. From repeated separations on this adsorbent, pure radioactive *a*-carotene and *β*-carotene (in solution) were obtained.

From the resulting solutions, the pigment concentrations were obtained from their light absorption spectra, and the radioactivity determined by plating aliquot portions. For plate counting, mylar end-window and Q gas G.M. tubes were employed; the planchets were prepared in 2 *N* acetic acid and dried in a stream of air. From these data the molar radioactivity was calculated. The differences in specific radioactivity can be an indication of the pathway of C^{14} in the biosynthesis of the pigments, higher values suggest earlier steps in biosynthesis while lower values would correspond to later steps. For the calculation

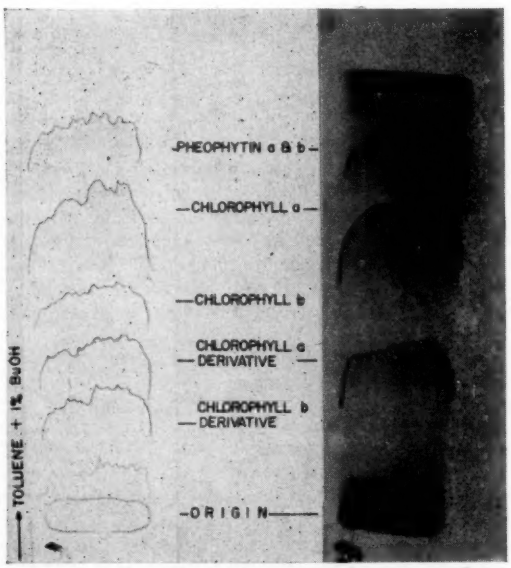


FIG. 1. Paper chromatogram (left) and autoradiogram (right) of green pigments from Scenedesmus after 1 hour photosynthesis with $C^{14}O_2$.

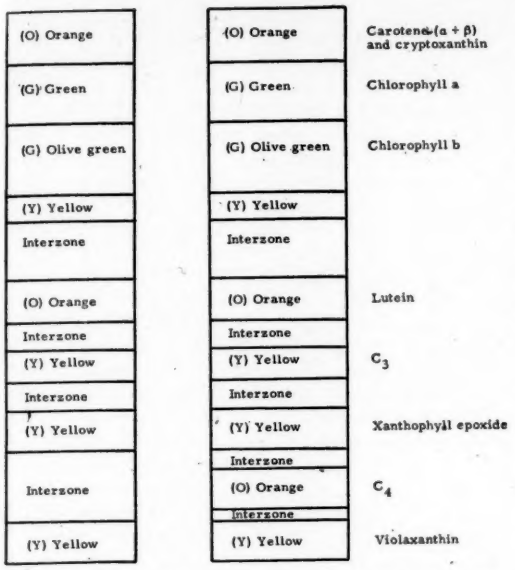


FIG. 2. Separation of the pigments of Chlorella (left) and Scenedesmus (right) in polyethylene powder columns with 80 % aqueous methanol.

of the concentrations from spectroscopic data we used the following molar extinction coefficients: Chlorophyll a: 91,000 at 663 mμ, 120,000 at 430 mμ; chlorophyll b: 51,500 at 645 mμ, 155,000 at 455 mμ; carotenoids: 135,000 at the wave length of maximal absorption. The calculated concentration figures contain a possible error of ± 5 %.

RESULTS

A paper chromatographic separation showed that pigments from Scenedesmus and Chlorella exhibit significant differences in their specific radioactivity after varying exposure times to light and C^{14}-radioactive bicarbonate. As an illustration of the varying radioactivity in algal pigments, figure 1 shows that among the green pigments, only the derivatives of chlorophyll a display enough radioactivity to blacken an autoradiogram, while chlorophyll b and its derivatives do not show up in the autoradiogram. The most striking point of a corresponding autoradiogram of a paper chromatogram of the carotenoids of Scenedesmus, was the lack of radioactivity in xanthophyll-epoxide even after 8.5 hours photosynthesis with $C^{14}O_2$.

The separation pattern of pigment extracts from both algal species in polyethylene columns with 80 % aqueous methanol as solvent is shown in figure 2. The only qualitative difference obtained in the pigment content of these algae is the carotenoid C4 which appears in Scenedesmus, but not in Chlorella.

From the simultaneous determination of the pigment concentrations of algae, one half of which was kept in the dark and the other half in the light for 30 minutes, the average figures for the concentrations of a series of carotenoids were determined by spectrophotometric methods. These values are shown in table I; most of the pigment concentrations show no significant differences between the light treated and the dark treated algae. The carotenoid violaxanthin, however, markedly increased in the dark as is shown in table II. Besides the actual change of concentration in the light and dark, the effect is much clearer in Chlorella than in Scenedesmus.

The molar radioactivity of the Scenedesmus pigments after 1 hour photosynthesis with $NaHC^{14}O_3$ is reported in table III. It is seen that chlorophyll a becomes radioactive considerably faster than chlorophyll b in green algae. In the series of carotenoids, 3 groups with high, medium, and low radioactivity can be distinguished. The 1st group consists of the 2 carotenes, the 2nd contains xanthophyll and violaxanthin, while the 3rd contains xanthophyll-epoxide and the unidentified mono- epoxy carotenoid, called C4 (possibly antheraxanthin). Each group of higher radioactivity would appear to be a biosynthetic precursor for the group with the next lower activity, while members of a given group would be in equilibrium with each other.

DISCUSSION

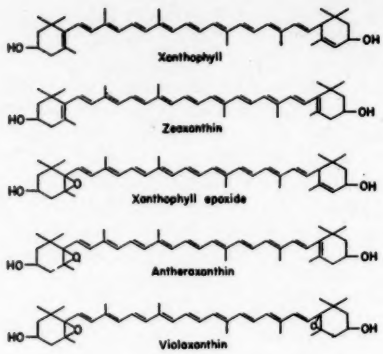
The very large differences between the specific activities of chlorophyll a and chlorophyll b, found in algae, confirm the earlier findings of Shylk and Godnev (14), who reported chlorophyll a to be more radioactive than chlorophyll b in higher plants even after 24 hours of exposure to $C^{14}O_2$. First of all, we may say unequivocally that these 2 substances are not

TABLE I

LIGHT DARK AVERAGE CAROTENOID CONCENTRATIONS IN ALGAE*

	CAROTENOID					
	α-CAROTENE	β-CAROTENE	XANTHOPHYLL	C3	XANTHOPHYLL-EPOXIDE	C4
Scenedesmus	24**	24**	130–160		47–53	61–68
Chlorella	39–41	24–30	150–190	4	44–51	

* Concentration of pigments in 10^{-8} mole per ml wet packed cells.
** Single determination only.



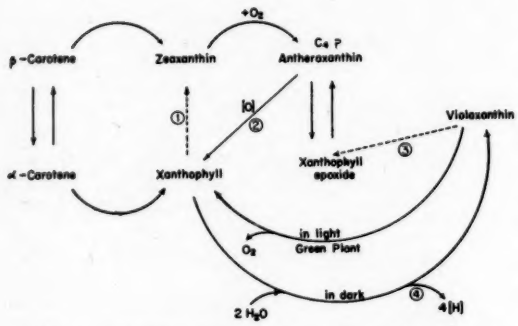


FIG. 3. Possible dynamic relationships between the carotenoids. 1) Rate-limiting in green plants. 2) [O] available as oxidant for other systems. 3) Possible slow step. 4) [H] ultimately for CO_2 reduction.

rapidly interconverted; beyond this we may suggest that chlorophyll a is a precursor of chlorophyll b, or else its turnover rate is very much higher, or both. It would appear that the precursor-product relationship is the more likely one in view of Shylk and Godnev's results (14), since they reported that the radioactive distribution for 3 fragments of the chlorophyll molecule, that is for the phytol, the methyl ester and the chlorin ring, is the same for both chlorophyll a and b.

The depression of violaxanthin concentration by light confirms the findings of Sapozhnikov (13), although we were not able to see the corresponding smaller percentage rise in the concentration of xanthophyll in our algae experiments. The proposed interrelationship between these 2 substances is strongly supported by the fact that their specific radioactivities are very nearly equal. Such a light dark interconversion between xanthophyll and the diepoxide, violaxanthin is indicated in figure 3. This scheme would require that the hydration of xanthophyll and its dehydrogenation to violaxanthin be dark, enzymatic reactions while the expulsion of molecular oxygen from violaxanthin giving back xanthophyll would be a light dependent transformation. A similar result would, however, be obtained if we supposed the dark, direct oxidation by molecular oxygen of xanthophyll and a photoreduction of violaxanthin. An unequivocal distinction between these two alternatives would be provided by the demonstration of isotopic oxygen content in violaxanthin isolated from plants which have been photosynthesized in the presence of H_2O^{18}.

The much lower specific activity observed in the intermediate oxidation levels represented by xanthophyll epoxide and the carotenoid (C4), possibly antheraxanthin, suggests that these are not intermediates in the interconversion between violaxanthin and xanthophyll. If the O_2 transport system proposed by Cholnoky et al (6, 7) is functioning as shown in figure 3, whereby zeaxanthin is oxygenated to antheraxanthin which may then give up its O_2 atom to some other substrate and return to xanthophyll, we would have a weak coupling between the xanthophyll-violaxanthin system and the xanthophyll epoxide-antheraxanthin system. It is possible that a slow leak from the violaxanthin directly into the epoxide might occur as shown in figure 3, step (3).

Finally, the very high specific radioactivity of the carotenes would implicate these compounds as precursors to all of the more oxygenated forms we have just discussed. This would be in keeping with the results of other laboratories (1, 5, 6, 7, 10) which have the more reduced forms in general, as precursors to the more oxidized forms.

TABLE II

VIOLAXANTHIN CONCENTRATION AFTER ONE-HOUR PERIODS IN LIGHT AND DARK*

CHLORELLA		SCENEDESMUS	
DARK	LIGHT	DARK	LIGHT
42.6	22.0	56.2	48.4
52.0	24.0	52.8	43.6

*Concentration of pigments in 10^{-8} mole per ml wet packed cells.

TABLE III

MOLAR RADIOACTIVITY OF SCENEDESMUS PIGMENTS AFTER ONE-HOUR OF PHOTOSYNTHESIS WITH $NaHC^{14}O_3$*

	millicuries per mole
Violaxanthin	74
C4	13
Xanthophyll-epoxide	6
Xanthophyll	78
a-Carotene	1130
	814**
β-Carotene	622
	700**
Chlorophyll a	147
Chlorophyll b	56

* Specific radioactivity: 11.1 curies per mole.
**Data from a previous 1 hour photosynthesis experiment with Scenedesmus.

SUMMARY

1. Light induces a decrease in the concentration of violaxanthin in the algae, Scenedesmus and Chlorella.

2. The specific radioactivity of a number of pigments has been determined for algae fed $C^{14}O_2$ for varying periods of time.

3. The specific radioactivity of chlorophyll a is nearly 3 times that of chlorophyll b; therefore these 2 substances are not involved in a rapid, reversible conversion.

4. The specific radioactivity of a group of carotenoids varies from 1000 for the carotenes, down to a value less than 10 for xanthophyll-epoxide.

5. The specific radioactivity of violaxanthin and xanthophyll are very nearly equal at 75.

6. It is suggested that violaxanthin and xanthophyll are rapidly interconverted in a light dark reaction, and that this reaction may be part of the O_2 evolution sequence in photosynthesis.

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STUDIES ON NITROGEN FIXATION AND PHOTOSYNTHESIS OF RHODOSPIRILLUM RUBRUM [1, 2]

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Nitrogen fixation in the photosynthetic bacterium *Rhodospirillum rubrum* has been described as an anaerobic, light dependent process (4, 7, 8, 9). Nevertheless, N_2 fixation by *R. rubrum* has not been studied in sufficient detail to permit one to answer the question as to how soon N_2 fixation stops after a period of illumination, or whether one might find conditions which would permit N_2 fixation to occur in the dark at rates that could be measured quite readily. Consequently, it was of interest to re-investigate the light requirement for N_2 fixation in greater detail with the help of a recording mass spectrometer (2) which permits one to follow N_2 uptake as well as photosynthetic and respiratory gas exchanges in an almost continuous manner. With this method it was thus possible to study the effect of light intensity on rates of N_2 fixation and photosynthesis in the same sample, and to investigate the effect of a number of substances on CO_2 and N_2 uptake in the light.

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[3] Kettering Foundation Fellow.

Materials and Methods

Rhodospirillum rubrum (Esmarch) Molisch (Sl isolated by Dr. C. B. van Niel) was maintained in stabs and in liquid cultures in a manner previously described (3, 4, 5). For experimental purposes, cultures were grown anaerobically in the light in 100 ml culture tubes containing 35 ml nitrogen-free, sterile culture medium (autoclaved for 20 min at 15 p.s.i.). The medium was that described by Gest, Kamen and Bregoff (4) except that it was neutralized with KOH instead of NaOH and that sources of combined nitrogen (glutamic acid and yeast extract) were omitted. Trace elements were supplied to give final concentrations in parts per million as follows: B, 0.5; Mn, 0.5; Mo, 0.3 (growth was extremely limited in nitrogen-free media containing only 0.01 ppm); Zn, 0.05; Cu, 0.02; Co, 0.02.

A gas mixture of 5 % CO_2 and 95 % N_2 was supplied under slight pressure at the inlet of the culture tube and was dispersed into a fine stream of bubbles by a sintered glass tube. Slow bubbling (5 to 10 ml gas/min) was found to be most satisfactory to avoid foaming. Inoculations were made with 3 ml of a culture grown in the above nitrogen-free medium for 48 to 72 hours. Little or no growth occurred in cultures which were not continuously supplied with CO_2. Since cultures supplied with glutamic acid under otherwise identical conditions also failed to develop in the absence of CO_2, this CO_2 requirement was not restricted to cultures dependent on N_2 as a nitrogen source.

Cultures grown on N_2 were incubated at 25 to 28° C and were illuminated by four 60-watt bulbs 30 cm from the center of the culture tube. For experimentation, 48-hour-old cultures (cell density 0.8 to 1.0 mg dry wt/ml) were harvested at 1000 × gravity in a refrigerated centrifuge and resuspended in 3 to 4 ml of fresh, nitrogen-free medium at pH 7.0 with added potassium phosphate giving a final concentration of 0.01 M phosphate. Two ml of this suspension were placed in a manometer vessel which could be attached to the leak housing of the mass spectrometer and immersed in a constant temperature bath at 28° C. The vessel was illuminated by a 1000-watt tungsten lamp, focused by 2 condensing lenses. Light intensities, varied with screens, were measured with a calibrated Weston light meter.

The method used for gas analysis in the present investigation was an adaptation of the one developed by Brown and coworkers (1, 2, 6) for the simultaneous measurement of gas exchange in respiration and photosynthesis. The mass spectrometer employed (Consolidated Engineering Company Model 21–201) was modified, as previously described, to permit periodic determinations of the partial pressure of gases in a manometer vessel attached to the mass spectrometer through an appropriate leak assembly. The manometer vessel itself could be shaken in a bath and maintained at constant temperature. The following masses were analyzed: 29 (N^{14}-N^{15}), 40 (A), and 44 (CO_2), by automatically cycling the ion accelerating voltage through the proper values. The cycling time was adjusted to obtain a reading for each mass every 1.8 or 3.6 minutes.

The use of mass 28 for the detection of changes in the partial pressure of N_2 was complicated by the fact that CO formed as a degradation product of CO_2 within the mass spectrometer contributed to the mass 28 peak. Rather than correct for this effect due to the presence of CO_2, N_2 enriched with N^{15} (30 atom % N^{15}) was used in the gas phase, and mass 29 determinations were employed to follow changes in the partial pressure of N_2.

Argon (mass 40) was included in the gas phase to provide a means for detecting any variations in the readings due to non-biological causes. Argon had

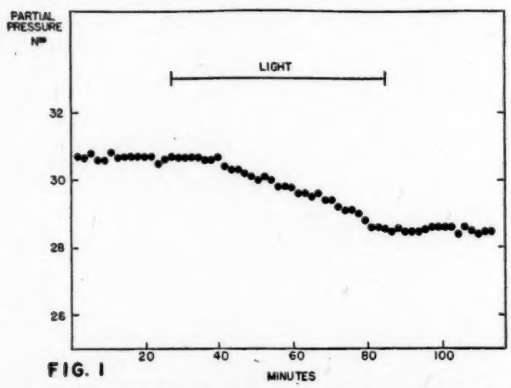

FIG. 1

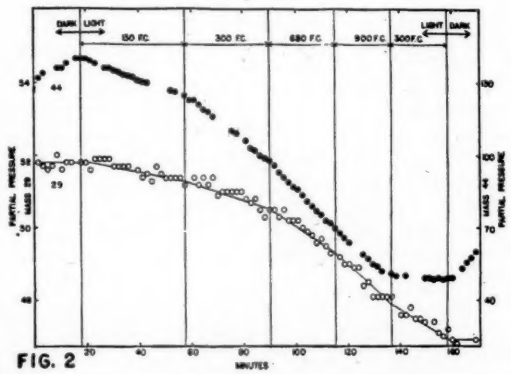

FIG. 2

FIG. 1. The effect of light on uptake of N_2 by *R. rubrum*. Light intensity: 1750 ft-c. The numbers on the ordinate of this figure and subsequent figures are in relative units. The following conversion factor must be applied to relate the ordinate for this figure to absolute units: partial pressure of mass 29 × 6.0 equals microliters of N_2.

FIG. 2. The effect of light intensity on uptake of CO_2 and N_2 by *R. rubrum*. Ordinate values: partial pressure of mass 29 × 11 equals microliters N_2; partial pressure of mass 44 × 6.0 equals microliters of CO_2.

been used for a similar purpose by Sisler and Zobell (12) in long term experiments designed to detect biological N_2 fixation.

Gas pressure in the manometer vessel was adjusted to atmospheric pressure (approximately 740 mm/Hg) at the start of each experiment.

Results

Light Relations: The results of one of the first attempts to follow N_2 fixation with a mass spectrometer are shown in figure 1. The suspension of Rhodospirillum was incubated in the manometer vessel with a gas phase consisting of 2 % N_2, 2 % CO_2, 2 % A and 94 % Helium. N_2 uptake is indicated by the decrease in the partial pressure of N_2 in the light; uptake of N_2 in the dark could not be established by the experimental method employed in any of the experiments which were performed.

It is apparent from figure 2 that the rates of N_2 and CO_2 uptake were related directly to light intensity. The metabolism of CO_2 in the latter part of the experiment was complicated by an effect which also was observed in suspensions kept at constant light intensity. Such suspensions showed an initial high rate of CO_2 uptake which decreased after a period of illumination (cf. fig 4, 6); this decrease in net CO_2 consumption could have been due to an increase in the rate of photofermentation (photoproduction of CO_2), however, the change in the photometabolism of CO_2 appeared to have little or no effect on the rate of N_2 uptake.

Inhibitors of Nitrogen Fixation: Kamen and Gest (7) reported that ammonium chloride inhibits N_2 fixation by *R. rubrum* and such inhibition also could be demonstrated with the mass spectrometer technique. An example is given in figure 3, where the addition of 0.2 ml of 0.05 M NH_4Cl (to give a final concentration of 4.5×10^{-3} M NH_4Cl) to a suspension of cells actively fixing molecular nitrogen produced complete inhibition after a lag period of 15 to 20 minutes; in different experiments this time lag could always be observed varying from 6 to 20 minutes.

With the analytic method described here it could be demonstrated that molecular hydrogen inhibits N_2 fixation by *R. rubrum*. A typical experiment is depicted in figure 4; a manometer vessel with a Rhodospirillum suspension was flushed with a gas mixture of 4 % N_2, 5 % CO_2, 89 % H_2 and 2 % Argon. Upon illumination N_2 uptake did not occur even though active CO_2 uptake could be observed. The atmosphere in the vessel then was displaced by a gas mixture of the same composition except for the replacement of H_2 by He. Upon subsequent illumination, active N_2 uptake could now be observed but there was little or no change in the rate of CO_2 uptake compared with the previous light period. Inhibition of N_2 fixation was complete at the H_2 and N_2 partial pressures used in this experiment. Partial inhibitions could be observed at lower tensions of H_2, for instance with a gas mixture of 45 % H_2 and 2.5 % N_2 (the remaining gases being CO_2, A and He) an inhibition of 60 % was observed against a suitable control. The details of the kinetics of the effect of H_2 on N_2 fixation by this organism remain to be worked out.

Molecular oxygen completely inhibits N_2 fixation by *R. rubrum* at relatively low partial pressures. An example is given in figure 5; in this experiment the initial gas phase contained 4 % N_2, 5 % CO_2, 2 % A and 89 % He with molecular oxygen present only in trace amounts, and under these conditions active N_2 uptake could be observed in the light. This gas phase was then displaced with a gas mixture containing approximately the same proportions of N_2, CO_2, A and He and in addition 4.1 % O_2 (the O_2 tension dropped to 3.8 % by the end of the light period as a result of respiration); upon subsequent illumination of the cell suspension N_2 uptake was completely suppressed. In other experiments, comparable to the one described here, the inhibition of N_2 uptake by 4 % O_2 could be reversed completely by making the system anaerobic again. Figure 6 demonstrates a partial suppression of N_2 fixation in the presence of a gas mixture containing 1.8 % O_2 giving an inhibition of about 45 %. The experiment also shows the reversal of this inhibition upon reduction of the partial pressure of O_2.

Discussion

The experimental findings of this paper support the observation that N_2 fixation by *Rhodospirillum rubrum* is essentially light dependent. Dark fixation of N_2 was not detectable by the methods employed in this study even in the period immediately after illumination.

The rates of N_2 fixation were strongly influenced by light intensity (fig 2). At low light intensities, rates of N_2 fixation and photosynthesis both were directly related to light intensity. Unfortunately, we were not able to obtain a good saturation curve for photosynthetic CO_2 uptake under the experimental conditions because of what appeared to be photofermentative production of CO_2. As a consequence a study of N_2 uptake at still higher light intensities, to establish a complete saturation curve, was not pursued at this time. However, at high light intensities the rate of N_2 fixation did not increase in a linear fashion with increase in light intensity and N_2 uptake most likely was saturating near or somewhat above the highest light intensity employed here. Further studies on the effect of light intensity on the rates of photosynthesis and N_2 fixation most likely should be carried out in some detail.

Although N_2 fixation by *R. rubrum* is essentially light dependent, Gest, Kamen and Bregoff (4) and Lindstrom, Newton and Wilson (9) have reported small amounts of fixation aerobically in the dark. In the experiments of these investigators, suspensions of *R. rubrum* were incubated for long periods of time (5 to 6 days) in a closed atmosphere containing N_2 enriched with N^{15}. Under these conditions the O_2 partial pressure undoubtedly dropped to a very low level. It is possible that the N_2 fixation which they observed took place at partial pressures of O_2 which were sufficient for oxidative metabolism, but insufficient to

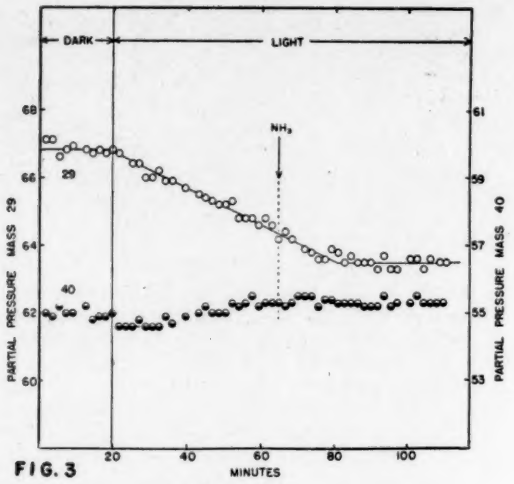

FIG. 3

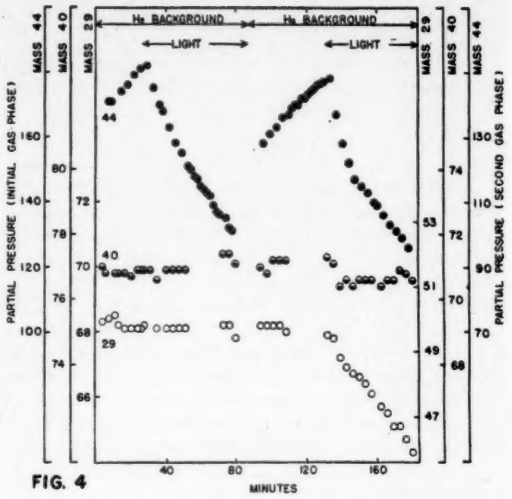

FIG. 4

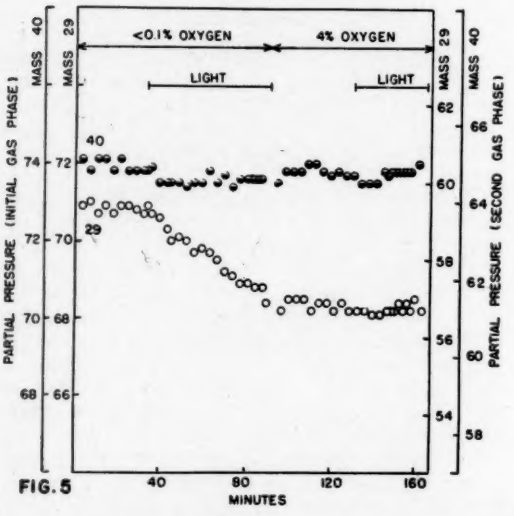

FIG. 5

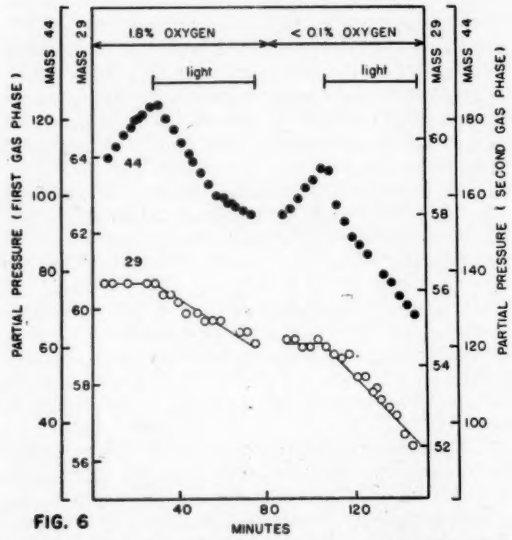

FIG. 6

FIG. 3. The effect of ammonium chloride on uptake of N_2 by *R. rubrum*. The side arm, containing 0.2 ml of 0.05 M NH_4Cl, was tipped at the time indicated. Light intensity: 1250 ft-c. The data for argon (mass 40) are included to indicate the type of variation commonly found in readings for the inert gas. Ordinate values: partial pressure of mass 29 × 11 equals microliters of N_2; partial pressure of mass 40 × 4.5 equals microliters of argon.

FIG. 4. The effect of molecular hydrogen on uptake of N_2 by *R. rubrum*. Light intensity: 1600 ft-c. Ordinate values: partial pressure of mass 29 × 6.9 equals microliters of N_2; partial pressure of mass 44 × 5.0 equals microliters of CO_2; partial pressure of mass 40 × 2.9 equals microliters of argon.

FIG. 5. The effect of low partial pressures of oxygen on uptake of N_2 by *R. rubrum*. Light intensity: 1150 ft-c. Ordinate values: partial pressure of mass 29 × 6.9 equals microliters of N_2; partial pressure of mass 40 × 2.9 equals microliters of argon.

FIG. 6. The effect of low partial pressures of oxygen on uptake of N_2 by *R. rubrum*. Light intensity 1600 ft-c. Ordinate values: partial pressure of mass 29 × 6.7 equals microliters of N_2; partial pressure of mass 44 x 5.0 equals microliters of CO_2.

inhibit N_2 fixation completely. Such low rates of N_2 fixation would be difficult to pick up with the method described here and the incorporation of N^{15} derived from N_2 over several days most likely is a more sensitive method for the detection of slow N_2 fixation in the dark.

When ammonium chloride was added to a cell suspension actively fixing N_2 a lag was noticed before inhibition of N_2 fixation became apparent. It may be of interest to study this lag time as a function of inhibitor and hydrogen ion concentration to determine whether the rate of penetration of ammonia was an important factor in this phenomenon.

Molecular hydrogen is a well known competitive inhibitor of N_2 fixation in a number of organisms (13), but attempts to demonstrate inhibition in the anaerobic Clostridium have not been successful (11). Therefore it has been assumed by some investigators that molecular hydrogen is not inhibitory to N_2 uptake by anaerobic N_2 fixers (13, 14). Pengra and Wilson, however, have shown recently that H_2 is inhibitory to the anaerobic N_2 fixing system of *Aerobacter aerogenes* (10). Nitrogen fixation by *R. rubrum* also is inhibited by molecular hydrogen, although the nature of this inhibition (competitive or non-competitive) has not been established.

It is of interest that N_2 fixation by Rhodospirillum is comparable to that of many organisms carrying out dark fixation of N_2 in its behavior toward inhibitors, pointing to the likelihood that the basic mechanism of N_2 fixation in these organisms must be rather similar. The fact, however, that N_2 fixation in *R. rubrum* is so strongly light dependent favors the assumption that the intermediate required for the initial fixation of N_2, or for the elaboration of early products of N_2 fixation, must be close to the primary photochemical products and that these intermediates never accumulate in sufficient amounts to bring about an appreciable dark fixation of N_2.

Summary

1. Metabolic gas exchanges involving CO_2, N_2 and O_2 by *Rhodospirillum rubrum* have been studied using a recording mass spectrometer. This technique is convenient for following N_2 fixation over short experimental periods. The effect of changes in environmental conditions which may affect N_2 fixation can be observed within 5 to 10 minutes.

2. Earlier reports on the light dependence of N_2 fixation by *Rhodospirillum rubrum* have been confirmed, and it also has been demonstrated that fixation stops abruptly once illumination ceases. The rate of N_2 fixation is an almost linear function of light intensity at low light intensities, paralleling photosynthetic CO_2 uptake which proceeds at about 13 times the rate of N_2 uptake.

3. It has been established that molecular hydrogen reversibly inhibits the light dependent N_2 fixation in this organism.

4. Molecular oxygen also brings about a reversible inhibition of N_2 fixation. Four percent O_2 brings about complete inhibition of N_2 uptake.

The authors wish to express their appreciation to Dr. A. H. Brown for making the mass spectrometer facilities available. They also wish to express their appreciation to Dr. Brown and to Dr. N. E. Good for numerous helpful suggestions.

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PIGMENT CARRYING STRUCTURES OF THE PHOTOSYNTHETIC NON-SULPHUR PURPLE BACTERIUM RHODOPSEUDOMONAS SPHEROIDES[1]

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As observed under the electron microscope (6), aqueous extracts of photosynthetic bacteria contain grana-like structures; most probably, these organelles are built up of lamellae. Pardee, Schachman and Stanier (5) showed that the colored sediment obtained by centrifugation of aqueous extracts of *Rhodospirillum rubrum* consisted of the above-mentioned grana-like structures. Niklowitz and Drews (4) prepared ultra-thin sections of *Rhodospirillum rubrum*. Electron microscopical examination revealed that this photosynthetic bacterium contains lamellated structures. This was confirmed by Elbers, Minnaert and Thomas (2). On the other hand, Vatter and Wolfe (9) observed spherical organelles packed throughout the bacterial cell. These organelles were considered to be chromatophores. Vatter and Wolfe (9, 10) stated that light is essential for the formation of these chromatophores. The latter authors studied a number of photosynthetic bacteria, among which was *Rhodopseudomonas spheroides*.

Thus, the results seem to be conflicting. A private discussion, however, revealed that the age of the bacteria may be responsible for this discrepancy. Vatter and Wolfe used bacteria only a few days old, whereas Elbers et al studied 7- to 10-day-old cultures which were still in a photosynthetically active state.

Goedheer (3) studied polarization of bacteriochlorophyll fluorescence in *Rhodospirillum rubrum* and Chromatium. Since, be it only to a relatively small degree, polarization was found to occur, it was concluded that the pigment molecules are preferentially oriented. This conclusion favors the conception of the occurrence of flat lamellae rather than that of vesicles. In any case, if the bacteriochlorophyll molecules were attached to the surface of vesicles, these structures should be flattened in a preferential plane. It would be interesting to study polarization of bacteriochlorophyll in both young and aged cultures.

Though it seems likely, evidence that bacteriochlorophyll is connected with lamellar structures in some way or another is so far lacking. Working with *Hibiscus rosa sinensis*, Thomas, Post and Vertregt (8) obtained the indication that, in the living cell, the occurrence of chlorophyll is restricted to the grana lamellae. They used the Molisch reaction, i.e. silver nitrate reduction in the presence of excited chlorophyll, as a tool for localization of this pigment. However, because of the small dimensions of the bacterial photosynthetic apparatus, the silver nitrate method cannot be expected to yield reliable results. For this reason, attempts were made to localize the photosynthetic pigments within the bacterial cell by studying ultra-thin sections of the colored fraction of Rhodopseudomonas homogenates under the electron microscope. The present paper shows the results of this attempt.

MATERIALS AND METHODS

Rhodopseudomonas spheroides cells, grown in a 1 % peptone and 0.5 % NaCl medium, were collected from about 10-day-old cultures by centrifugation. They were resuspended in 0.1 M TRIS buffer of pH 8.0. This suspension was sonicated at 7 Kc in a magnetostriction oscillator for 30 minutes. During disintegration, the suspension was cooled with tap water. The colored material was separated by differential centrifugation and repeatedly washed in a Spinco ultracentrifuge with the buffer solution. Finally, the clear and colorless supernate was decanted and the sediment, which seemed to consist entirely of colored matter, was used for examination. A detailed description of this procedure will be published by Bril (1).

The sediment was fixed with 1 % osmium tetroxide solution, phosphate-buffered at pH 7.2, and embedded in methylmethacrylate as described by Elbers et al (2). Preparation and sectioning were carried out by the Technical Physical Service T.N.O. and T.H., Delft, who also made the electron micrographs. The thickness of the sections, prepared with a Philips ultramicrotome, was about 200 Å.

RESULTS

Figure 1 shows an electron micrograph of a section through sonicated and purified colored matter from *Rhodopseudomonas spheroides*. Evidently, this disintegrated fraction consists almost entirely of lamellar material. As a rule, the cross-sectioned lamellae show up as curved lines. Since one never is certain whether a lamella was oriented in a plane perpendicular to the plane of cutting, it is impossible, to determine the lamellar thickness without doubt. It can only be stated that, probably, the thickness of the lamellae is about 30 Å.

As far as the lamellae occurred in a plane parallel to the plane of cutting, or under a slight angle to it, they can be observed as homogenous half-tones. Globular cytoplasmatic matter seems to be nearly, if not totally, absent in the preparation. Since the occurrence of colored material in the homogenate is re-

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stricted to the fraction under consideration, it may be concluded that, most probably, the photosynthetic pigments occur in, or at the surface of, lamellae in the bacterium studied.

Discussion

Instead of grinding, sonic treatment was chosen as a means to disintegrate the bacteria. This was done in order to obtain a high degree of separation of the structural elements from each other, and, thus, to enable thorough washing of the pigment containing fraction. However, this method of disintegration is likely to cause a more or less serious distortion of the material studied. As a consequence, it is impossible to decide whether the curling of the lamellae, as shown in figure 1, should be considered an artifact or representation of the original shape. Moreover, distortion of the isolated lamellae might also be caused by the fixation technique which was used. For these reasons, the above result is not difinitive with regard to establishment of the shape of the photosynthetic organelle in the living cell.

The data under consideration indicate that, in the photosynthetic bacterium *Rhodopseudomonas spheroides*, the pigments are attached to lamellae. Such a situation probably occurs in higher plants (8). Thus, the present investigation suggests that the structural units of the photosynthetic apparatuses of both the bacterium and the higher plants are similarly constituted. This suggestion favors the conception (7) that arrangement of chlorophyllous pigments on lamellar structures is essential for the functioning of these pigments in photosynthesis.

Summary

1. Ultra-thin sections of the pigment-containing fraction of sonically disintegrated cultures of *Rhodopseudomonas spheroides* were studied under the electron microscope.

2. This fraction was found to consist of lamellar material of a thickness of, probably, about 30 Å.

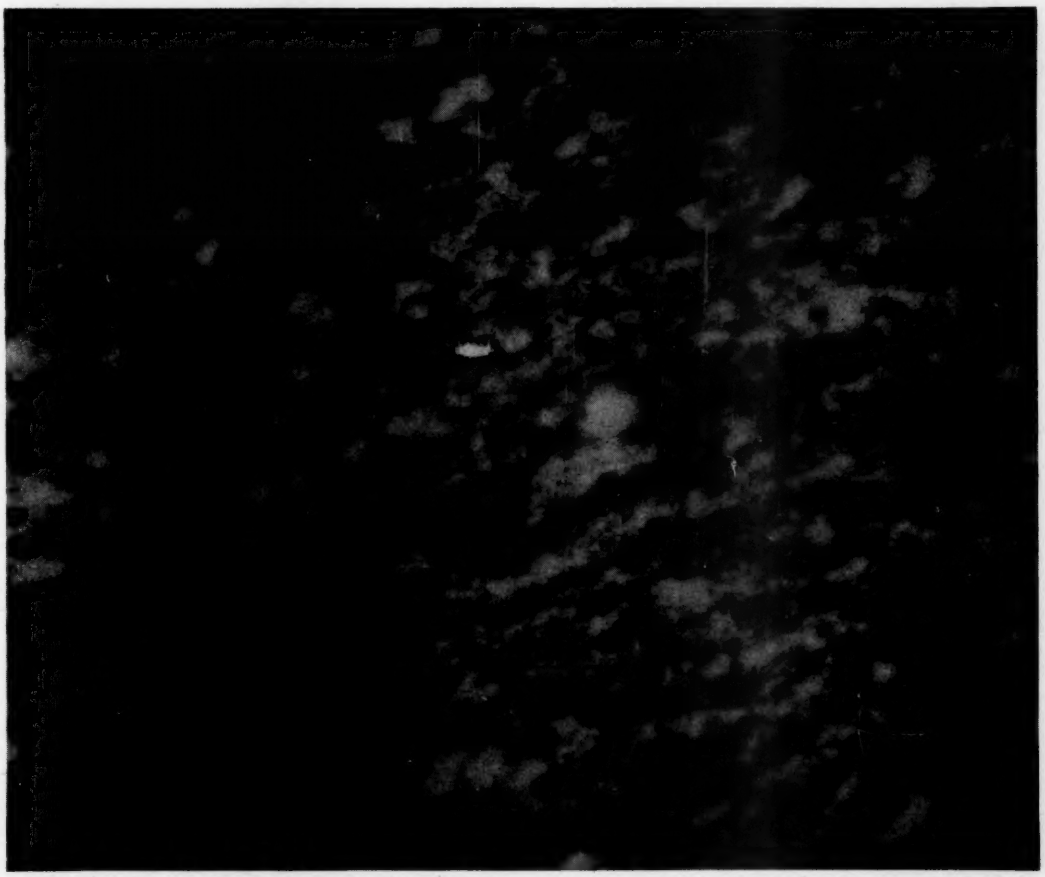

Fig. 1. Electron micrograph of a section through the pigment-carrying fraction from a homogenate of *Rhodopseudomonas spheroides.*

3. It was concluded that the photosynthetic pigments of the studied bacterium are likely to be attached to lamellae.

Thanks are due to Dr. J. A. Niemeyer for placing the magnetostriction oscillator at our disposal, and to Mr. C. Bril for preparation and purification of the bacterial extract.

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FORMATION AND BLEACHING OF CHLOROPHYLL IN ALBINO CORN SEEDLINGS[1]

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In 1951 Koski and Smith (5) found that albino seedlings of corn formed protochlorophyll and chlorophyll equally as well as normal plants. These albinos lacked the ability to accumulate chlorophyll, however, because the chlorophyll was bleached by continued illumination. Several factors were suspected of causing the instability of the chlorophyll, and this paper presents an examination of these factors.

Granick (3) observed that a Chlorella mutant which formed only protochlorophyllide (i.e., phytyl-free protochlorophyll) bleached in the light. He suggested that the loss of pigment was caused by the lack of the phytyl group. Loeffler (6, 11) and Wolff and Price (15) discovered independently that normal seedlings grown in the dark formed both protochlorophyll and protochlorophyllide, which they transformed to chlorophyll a and chlorophyllide a in the light and subsequently esterified the chlorophyllide a to chlorophyll a. If, in accordance with Granick's suggestion, the albino plants contained protochlorophyllide and were unable to completely esterify (phytylate) the chlorophyllide formed therefrom, this might be a cause of albinism. For this reason a number of albino plants were examined for their esterifying ability.

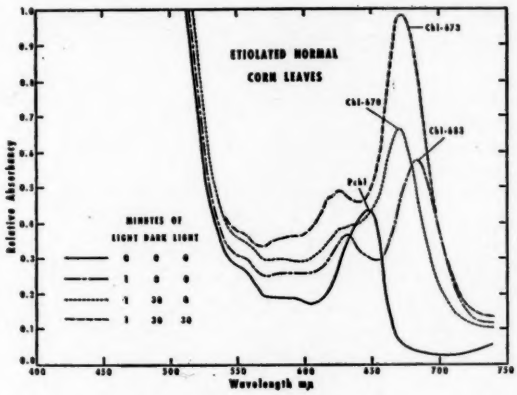
Another suspected cause of bleaching was that albino plants did not stabilize the chlorophyll after it was formed. Shibata (8) discovered that in normal plants the chlorophyll first formed from transformation of protochlorophyll had an absorption maximum at about 684 mμ. When the plants were allowed to stand in the dark the absorption maximum of this chlorophyll changed to about 670 mμ. Since the chlorophylls extracted from these two forms showed the same absorption spectrum in ether the difference between them was attributed to the variation in the relation of pigment to carrier rather than to the pigment molecules themselves. It was surmised that this change in chlorophyll absorption might signify the stabilization of the newly formed chlorophyll. A number of albinos have been examined in respect to this post-illuminative spectrum shift to determine whether it can be correlated with chlorophyll bleaching.

A third factor examined was the relation of carotenoid content to bleaching. Willstätter and Stoll (14) suggested that the function of the yellow pigments in leaves was to protect the chlorophyll from bleaching. Cohen-Bazire and Stanier (1) have recently suggested "that the carotenoid pigments characteristically associated with the photosynthetic apparatus perform an essential physiological function, by protecting the cell from the deleterious effects of chlorophyll-catalysed photo-oxidations." Koski (cf. 13) found, however, that one chlorophyll-deficient mutant of corn, Golden 1, contained a large quantity of yellow pigment and yet bleached. It seemed worthwhile, therefore, to examine other corn mutants to determine whether the yellow pigments afford any protection against chlorophyll bleaching.

[1] Received April 1, 1959.

Materials and Methods

Esterification Experiments: The seedlings, both normal and albino, were grown in complete darkness except for the weak green or blue light used during the watering, picking and handling of the leaves [cf. Koski (5)]. After being harvested, 3 equal portions, weighing from 2.5 to 3.5 g, were taken from each lot of leaves and stored in a dark cold-room until they were used. One portion of leaves was extracted without being illuminated, another was illuminated and then immediately extracted, and the 3rd was illuminated and stored over night in the dark cold-room before being extracted. Illumination consisted in exposing the leaves for 20 minutes to light of 17 ft-c intensity from a fluorescent lamp. Each lot of leaves was cut into about 5-mm lengths and extracted in a Waring blendor successively with 25, 20, and 20 ml of 80 % acetone. The pigments were quantitatively transferred to ether [cf. Mackinney (7)] and the solution brought to 25.0 ml with ether (solution 1, total pigment) and the spectrum automatically recorded with a Beckman DK-2 spectrophotometer. Solution 1 was then extracted with 10, 8, and 7 ml portions of 0.1 M sodium bicarbonate in 25 % acetone and then with 10 ml of dilute sodium chloride solution. The ether solution was brought to 25.0 ml with ether (solution 2, neutral fraction). The alkaline extracts and salt solution were combined and extracted with 15 ml of ether while being treated with 18 to 25 ml of 0.1 M tartaric acid, added in 8 to 10 portions and shaken gently after each addition. The ether layer was removed quantitatively and the aqueous layer extracted with 10 ml of ether. The ether extracts were combined and diluted to 25.0 ml with ether (solution 3, acidic fraction). The absorption spectrum of each solution was recorded and the percentage of the total pigment recovered in the neutral, "N," and acidic fractions, "A," of tables I and II calculated. The columns headed "μg/2.5 g" mean that the weight of pigment in micrograms is in reference to 2.5 g fresh weight of leaves.



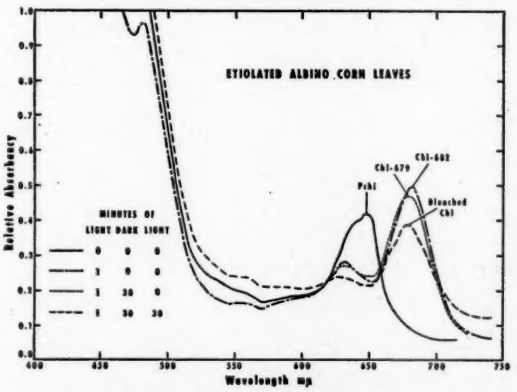


Fig. 1 (*top*). Changes produced in the absorption spectrum of a live etiolated normal corn leaf by the different light-dark treatments shown.

Fig. 2 (*bottom*). Changes produced in the absorption spectrum of a live etiolated albino corn leaf by the different light-dark treatments shown.

Post-illuminative Spectral Shift: The leaves were grown in the dark as has already been described. The albino leaves were harvested in blue light so as to be visibly distinguishable from their normal sibs. They were cut into 1- or 2-cm lengths, placed in the opal-glass apparatus of Smith, Shibata and Hart (12), and their absorption spectra recorded with the Beckman DK-2 spectrophotometer. The sequence of dark and light treatments used in handling the leaves is shown in figures 1 and 2. The leaves were illuminated with light of about 200 ft-c intensity from an ordinary frosted-bulb incandescent lamp while remaining in the holder and without being removed from the cell compartment of the Beckman instrument. This technique was adopted to avoid variation in absorption due to change in position of the leaves. The wave lengths and heights of the absorption maxima resulting from each treatment were measured and are reported in table III, column k.

Carotenoid Content: The absorption maxima of the carotenoid pigments, in the spectrophotometric recordings just described, lie close to 480 mμ. The absorbancies at this wave length were measured and are tabulated in table III. For comparative purposes, this value is divided by the height of the maximum near 647 mμ due to protochlorophyll. This quotient is given in table IV, column h'.

Results and Discussion

The results of the esterification experiments are shown in tables I and II. The age is the number of days from planting to harvest. The recovery, R, is the sum of N and A. The percentages of transformation, T, of the protochlorophyll-like pigments to chlorophyll-like pigments were obtained from the absorption spectra of the total converted pigments

(Solutions no. 1) according to the method of Koski (4). [cf. Smith and Benitez (10)].

In table II, "Mutant designation" refers to the code number of mutant assigned by donors of the seed. The headings "Not illuminated," "Illuminated-no standing," and "Illuminated-with standing" refer to the results obtained under the 3 light-dark sequences described under Esterification Experiments. For convenience in comparing results, the recovery was arbitrarily brought to 100 % and the percentages of neutral and acidic components adjusted accordingly (last line of table I).

The results in table I indicate that through photoconversion more acidic and less neutral pigment was obtained than existed in the leaf before illumination, an indication that hydrolysis occurs during the photochemical transformation. During an overnight sojourn in the dark the neutral pigment increased, which finding agrees with the observations of Loeffler, and of Wolff and Price, already referred to.

Comparison of these results with those obtained with albinos (table II) shows that initially most of the albinos have nearly the same percentages of neutral and acidic forms of the pigment as normal leaves. One exception is W-alb-7752 which has only a little of the acidic form. The albinos, as well as normal plants, hydrolyze the neutral esterified pigment during transformation. Most of the albinos fail, however, to esterify the acidic form on standing in the dark subsequent to being illuminated. Mutant Vp_5 is an exception to this and, significantly, it contains no appreciable amount of carotenoid. This implies that no connection exists between the carotenoids and esterification with a phytyl group, presumably derived from carotenoid [cf. Smith (9); Frank (2)]. The lack of phytyl cannot be the sole cause of albinism since one mutant, Vp_5 (and possibly W-alb-8852), esterfies its chlorophyllide and yet bleaches in the light.

In figures 1 and 2 are depicted the effects of illumination on the spectra of live etiolated normal and albino leaves. Both contain protochlorophyll-like pigments (continuous line) which they convert to chlorophyll-like pigments having an absorption peak near 683 mμ (dash-dot line). On standing in the dark at room temperature for 30 minutes the position of the chlorophyll absorption changes in the normal

TABLE I

NORMAL ETIOLATED CORN LEAVES: THE CONTENT OF NEUTRAL AND ACIDIC PROTOCHLOROPHYLL- AND CHLOROPHYLL-LIKE PIGMENTS IN ILLUMINATED AND UNILLUMINATED SAMPLES

	NOT ILLUMINATED				ILLUMINATED—NO STANDING					ILLUMINATED—WITH STANDING				
AGE DAYS	WT μG/2.5G	R %	N %	A %	WT μG/2.5 G	R %	N %	A %	T %	WT μG/2.5 G	R %	N %	A %	T %
16										8.2	97	86	11	80
										7.9	91	78	13	68
16—18	14.3	94	62	32	11.9	78	41	37	81	10.3	99	87	12	79
										11.9	94	85	9	82
15	18.5	83	71	12	13.6	90	80	10	90					
13	15.2	94	81	13	11.8	82	57	25	81					
14	17.0	80	69	11	13.9	79	61	18	79					
17					13.0	89	68	21	80	11.9	95	90	5	78
18										9.7	94	89	5	78
Average		88	71	17		84	61	22	82		95	86	9	78
Mean dev from mean		±3.7	±3.9	± 5		±2.5	±6.4	±6.1	±4		±1.3	±1.7	±1.4	±4
Adjusted		100	81	19		100	73	26			100	91	9	

TABLE II

ALBINO ETIOLATED CORN LEAVES: THE COMPARISON OF VARIOUS ALBINO CORN MUTANTS WITH RESPECT TO THEIR CONTENT OF NEUTRAL AND ACIDIC PROTOCHLOROPHYLL- AND CHLOROPHYLL-LIKE PIGMENTS IN ILLUMINATED AND UNILLUMINATED SAMPLES

		NOT ILLUMINATED				ILLUMINATED—NO STANDING					ILLUMINATED—WITH STANDING				
MUTANT DESIGNATION	AGE DAYS	WT μG/2.5 G	R %	N %	A %	WT μG/2.5 G	R %	N %	A %	T %	WT μG/2.5 G	R %	N %	A %	T %
W-alb-6474	10	26.8	92	64	28	20.5	84	58	26	74	20.6	91	64	29	81
W-alb-7748	13	32.9	92	70	22	22.8	81	52	29	72	21.2	72	38	34	69
W-alb-7752	11	25.1	94	87	7	19.1	85	65	20	76	13.7	90	72	18	79
Vp_5						14.0	82	69	13	79	15.9	93	90	4	78
lw_3 lw_4	10	27.0	89	69	20	19.2	86	55	29	79	20.6	88	57	31	79
											16.5	83	44	39	87

TABLE III

SPECTRAL PROPERTIES OF ETIOLATED NORMAL AND ALBINO CORN SEEDLINGS BEFORE AND AFTER VARIOUS EXPOSURES TO LIGHT

	MUTANT	PROTOCHLOROPHYLL 0-0-0 [2]		CHLOROPHYLL 1-0-0 [2]		CHLOROPHYLL 1-30-0 [2]		CHLOROPHYLL 1-30-30 [2]		CAROTENOID
NO.	REF. NO. [3]	λ MAX Mμ	A	λ MAX Mμ	A	λ MAX Mμ	A	λ MAX Mμ	A	A λ 480
a	b	c	d	e	f	g	h	i	j	k
1	1a	647	0.222	680*	0.305	680	0.300	676	0.085	0.770
2	1b	647	0.172	679	0.238	677	0.230	677	0.132‡	0.580
3	"	646	0.263	680	0.324	679	0.315	680	0.170	>0.920
4	1c	648	0.240	678*	0.300	677	0.310	677	0.210	0.630
5	2	646	0.275	679*	0.320	671	0.325	672	... [1]	> 1
6	3a	646	0.325	678	0.388	678	0.402	678	0.160	0.615
7	3b	645	0.175	680*	0.210	666	0.240	668	0.350	> 1
8	4a	640**	0.405	677	0.480	677	0.490	678	0.388	0.600
9	4b	644	0.498	678	0.630	678	0.640	682	0.380	0.720
10	4c	646	0.435	680	0.560	678	0.600	681	0.298	0.730
11	5†	647	0.036	682	0.040	668	0.042	665	0.009	0.130
12	6	648	0.220	677*	0.265	667	0.272	667	0.170	0.200
13	"	645	0.215	676	0.195	669	0.195	669	0.125	0.288
14	7	648	0.165	679	0.228	668	0.250	667	0.183	0.340
15	8	646	0.360	682	0.445	678	0.415	678	0.330	0.980
16	9	644	0.265	680	0.370	676	0.370	677	0.180	0.630
17	10	647	0.420	683	0.565	670	0.645	671	0.980	> 2

* 30 seconds of illumination rather than 60 seconds.

** Broad maximum the exact position difficult to assess.

† Sample very thin; accuracy poor.

‡ 15 minutes of illumination.

[1] Abnormal behavior on long illumination.

[2] Light-dark sequence used cf. figures 1 and 2.

[3] Mutant reference numbers refer to the designations given the various mutants: 1a, W-alb-6474 : 54-6506-33xn; 1b, W-alb-6474 : 54-6506-34xn; 1c, W-alb-6474 : 54-6506-102x; 2, W-alb-7716 : 54-6501-91xn; 3a, W-alb-7752 : 54-6538-1xn; 3b, normal plants from 3a seeds; 4a, W-alb-7748 : 54-6622-9xn; 4b, W-alb-7748 : 54-6622-10xn; 4c, 7748 : 54-6622-3x; 5, Vp_5 : 53-5171-14x; 6, Vp_5 : 53-5168-5/5189-2; 7, Vp_9 : 54-6670-59x; 8, lw_3lw_4 : 54-6588-1xn; 9, lw_3lw_4 : 54-6589-3xn; 10, normal yellow dent corn.

leaf to about 670 mμ whereas only a slight shift occurs in the albino leaf (dotted line). On continued illumination the chlorophyll peak of the normal leaf is enhanced but that of the albino leaf is depressed (dashed line). On 1 curve, the dot-dash line of figure 2, the carotenoid maximum at 480 mμ is evident.

The measurements taken from the absorption curves of the normal and albino mutants are collected in table III. Columns c, e, g, and i give the wave lengths of the absorption maxima of the green pigments after each treatment, and columns d, f, h, and j the corresponding absorbancies (or optical densities defined by $\log_{10} \frac{Io}{I}$). Column k presents the absorbancies at 480 mμ representing carotenoid pigments.

The wave lengths of the protochlorophyll maxima, column c, are very consistent, 646.3 ± 1.1 mμ, among the different mutants, except 4a. This mutant had such a broad maximum that its exact position was difficult to assess and was not included in the averages. After from 0.5 to 1.0 minutes of illumination, the positions of the chlorophyll absorption maxima were consistent, 679.4 ± 1.4. After standing in the dark, however, the position was 674.2 ± 4.8 which showed a marked average deviation from the mean which correlates with the conspicuous variation of the post-illumination shift found for the different samples (table IV column e').

Table IV sets forth various ratios and differences derived from the experimental data of table III. In column c' the quotients of the absorbancies of chlorophyll newly formed (f, table III) to absorbance of the protochlorophyll, d, is in every case but one, line 13, greater than 1. Some of the mutants show values higher than the normals, lines 7 and 17. In column d' the ratio of the absorbancies after standing for 30 minutes in the dark to those existing immediately after illumination is close to 1.0 for all the albinos but no. 7 (line 14) and are all less than the values for normal leaves, lines 7 and 17. In general the shifts in absorption maxima, column e', are small for the albinos as compared to normal leaves (lines 7 and 17) but exceptions to this are apparent (lines 11, 12, and 14). The quotients of the absorbancies of the chlorophyll peaks

before and after illumination (column f') give a measure of the bleaching caused by continued illumination: the lower the quotient the greater the bleaching. In every case the albino mutants have a quotient less than 1.0 and the normal plants a quotient considerably greater than 1.0. Column g' shows the changes in the positions of the absorption maxima of chlorophyll caused by continued illumination. No consistent trend in these values is apparent.

Column h' gives the quotients of the absorbancies at 480 mμ divided by the absorbancies of the protochlorophyll maxima. These values provide some measure of the carotenoids present. They are higher for the normal than for the mutant plants. Some mutants have a relatively high carotenoid content, however, and bleach nevertheless.

In columns i', j', and k' are given the percentages of the esterified pigments components from normal and mutant plants—the N values from tables I and II. These values were determined on mixtures of mutants from a given category and are bracketed to signify to what plants they belong. There is no obvious relationship between these values and the differences and ratios just discussed except, perhaps a slight correlation between the post-illumination shifts in the absorption maxima (column e') and the esterification of the protochlorophyllide.

Conclusion and Summary

All the albino corn seedlings examined form protochlorophyll and protochlorophyllide during germination and growth in the dark. They convert these pigments to chlorophyll and chlorophyllide on exposure to light. Most of the mutants subsequently esterify their chlorophyllide to only a slight extent as compared to normals but 1 mutant is as effective as normal plants in this respect. Most of the mutants show only a slight, if any, post-illumination shift of the chlorophyll absorption maximum yet one mutant is equal to normals in this regard. All mutants have less carotenoid pigment than normals; however, some are quite rich in this pigment. All mutants bleach with continued illumination.

It may be concluded therefore that neither esterification ability, post-illumination shift in absorption spectra, nor carotenoid content is solely responsible for the stability or instability of chlorophyll in leaves.

Table IV

Comparison of Etiolated Normal and Albino Corn Seedlings in Respect to Different Factors Derived from Data in Table III

No.	Mutant ref. no.[3]	f/d	h/f	e–g Δλ max mμ	j/h	g–i Δλ max mμ	k/d	Esterified Pchl %	Esterified First chl %	Esterified Stored chl %
a'	b'	c'	d'	e'	f'	g'	h'	i'	j'	k'
1	1a	1.38	0.98	0	0.28	−4	3.48			
2	1b	1.39	0.97	2	0.57	0	3.37	64	58	64
3	"	1.23	0.97	1	0.54	+1	>3.50			
4	1c	1.25	1.03	1	0.68	0	2.62			
5	2	1.16	1.02	8	...[1]	+1	>3.64			
6	3a	1.19	1.04	0	0.40	0	1.89	87	65	72
7	3b	1.20	1.14	14	1.46	+2	5.70			
8	4a	1.19	1.02	0	0.79	+1	1.48			
9	4b	1.27	1.02	0	0.59	+4	1.45	70	52	38
10	4c	1.29	1.07	2	0.50	+3	1.63			
11	5†	1.11	1.05	14	0.21	−3	3.60			
12	6	1.20	1.03	10	0.63	0	0.91	...	69	90
13	"	0.91	1.00	7	0.64	0	1.32			
14	7	1.38	1.10	11	0.73	−1	2.06			
15	8	1.24	0.93	4	0.80	0	2.72			
16	9	1.40	1.00	4	0.49	+1	2.38	69	55	57
17	10	1.35	1.14	13	1.52	+1	> 5	71	61	86

† Sample very thin; accuracy poor.

[1] Abnormal behavior on long illumination.

[3] Mutant reference numbers refer to the designations given the various mutants: 1a, W-alb-6474 : 54-6506-33xn; 1b, W-alb-6474 : 54-6506-34xn; 1c, W-alb-6474 : 54-6506-102x; 2, W-alb-7716 : 54-6501-91xn; 3a, W-alb-7752 : 54-6538-1xn; 3b, normal plants from 3a seeds; 4a, W-alb-7748 : 54-6622-9xn; 4b, W-alb-7748 : 54-6622-10xn; 4c, 7748 : 54-6622-3x; 5, Vp_8 : 53-5171-14x; 6, Vp_5 : 53-5168-5/5189-2; 7, Vp_9 : 54-6670-59x; 8, lw_3lw_4 : 54-6588-1xn; 9, lw_3lw_4 : 54-6589-3xn; 10, normal yellow dent corn.

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ON THE MASS CULTURE OF ALGAE. II. YIELD AS A FUNCTION OF CELL CONCENTRATION UNDER CONTINUOUS SUNLIGHT IRRADIANCE [1]

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The considerable effort which has been directed toward the mass culture of algae under sunlight illumination (15) makes clear the critical problem of achieving maximum yields or maximum efficiency in use of available solar radiation. Measurements by Kok (4) and Oorschot (10) on total cell synthesis by Chlorella under low illuminance at 589 mμ may be interpreted as showing a 20 $\pm$ 2% efficiency. Unpublished experiments of our laboratory, using entirely different techniques, show a maximum efficiency of 19 $\pm$ 1% under similar illumination. In contrast, observed yields in outdoor mass cultures cited by Tamiya (15) allow estimates of only 3 to 7% for efficiency of conversion of the visible or photosynthetically usable fraction of solar energy. A major fraction of the discrepancy has been attributed to the consequences of light saturation.

Estimate of performance of a dense algal culture under sunlight illumination may be visualized graphically from figure 1. Curve A is a characteristic irradiance curve for a very thin culture of Chlorella with rate measured in terms of apparent photosynthesis or specific growth rate. Its essential feature is that light saturation occurs at a low value, I_s, which is 1/20 to 1/10 of the irradiance of maximum sunlight. Curve B shows the expected decrease in irradiance within a dense culture. The abscissa is taken conveniently as Cl (cell concentration $\times$ depth) and a value of I_s on the ordinate is indicated at 1/20 of incident irradiance. Consider a cell at the upper surface of the culture (Cl = 0). It absorbs radiation at a rate proportional to $I_o = 100$ but works only at a rate proportional to $I_s = 5$. The argument may be repeated for all other values of Cl with the following result. Of the total light absorbed, measured by the area under curve B, only a fraction measured by the shaded area is used with maximum efficiency. The argument has been taken from a previous and more extensive treatment (6); it has been subjected to more elegant mathematical structure by Tamiya et al (16) for a hyperbolic irradiance curve and by Oorschot (10) for Blackman, hyperbolic, and logarithmic irradiance curves. The only essential difference between the 3 treatments lies in the shape assigned to the irradiance curve (fig 1, curve A).

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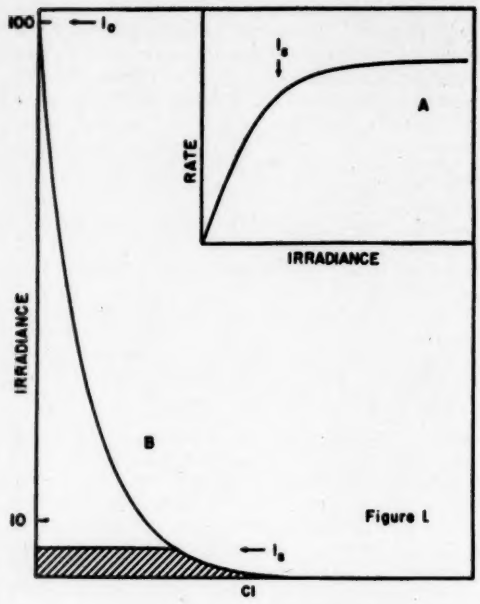
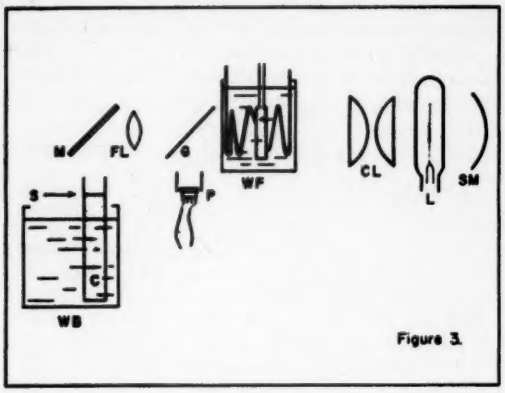
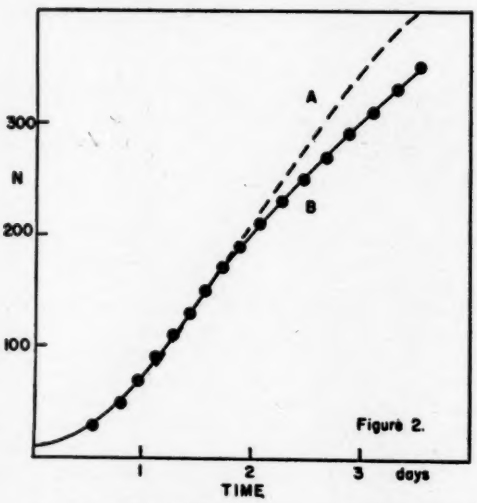






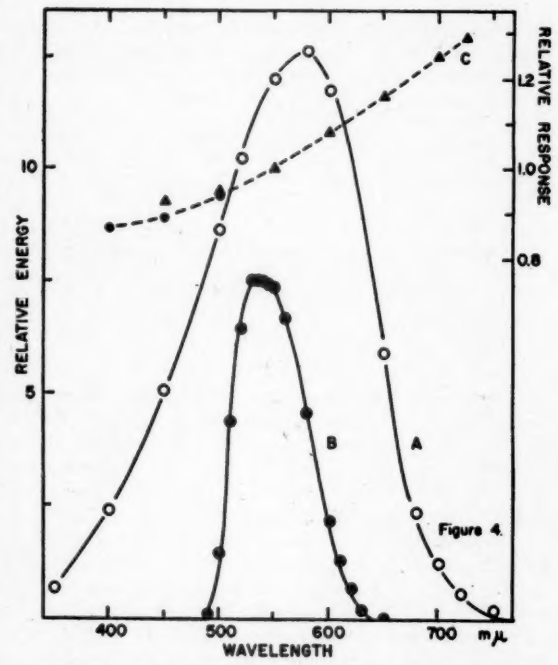


FIG. 1. Graphical description of expected performance of a dense algal culture under sunlight illumination. Curve A, a diagrammatic irradiance curve of rate of photosynthesis showing I_s, the estimated minimum irradiance for light saturation. Curve B, decrease in irradiance in the culture as a function of Cl, cell concentration × depth. See text.

FIG. 2. Growth curves, quantity of cells N vs. time, for a batch culture. Curve A, as expected from previous treatments. Curve B, as predicted from the present work, integrated from figure 6 A.

FIG. 3. The optical arrangement and culture described in the text.

FIG. 4. Spectral energy and response curves. Curve A, radiation used for the growth and photosynthesis measurements. Curve B, green radiation used for photosynthesis measurements. Curve C, spectral response of the Ektron lead sulfide cell from data provided by Eastman Kodak Co.; points ▲▲ denote check data obtained by comparison of the Ektron cell with a vacuum thermopile using a B and L grating monochromator giving 31 mμ total band width.

From the argument presented above the basic difficulty is clear: the chloroplast of Chorella is geared to efficient use of an energy flux far lower than that of full sunlight. The argument might be extended to inquire whether this is an inherent general characteristic of chloroplast structure and whether higher plant chloroplasts are subject to the same problem. For algae several ways of circumventing losses due to light saturation have been suggested. Advantageous flashing light effects can be achieved by turbulence of culture (2, 11) but the time constants are so small that the power requirements to produce necessary turbulence appear excessively high. Search for algae with high values of I_s has been made but has not been successful except for strains tolerant to higher temperature (12, 13); even here no remarkable gains in yield were found in practical tests (15). A third attack might be made by some physical method of attenuating the high irradiance of sunlight without energy loss, i.e., by spreading it out over a greater area as viewed by cells of the culture. An attractive possibility is the use of diffusing cones, inverted base up, and projecting into a dense culture (7). The illuminated area of algal suspension then can be made 10 times as great as the surface area of the cone bases exposed to sunlight irradiance with a possible 10 times decrease in I_o.

In order to test the diffusing cone we designed an optical system which would deliver continuously a visible irradiance equivalent to the limit case of full sunlight. The intent was to determine yields or efficiencies with and without the cone in place. In order to estimate yield we initially followed the growth curve of a culture started from a small inoculum. For example the maximum slope of the growth curve such as figure 2 has been taken as the maximum yield in mg/day (3). We found 3 objections to this method. It did not permit the precision desired. Sampling losses introduced changes in culture volume or awkward dilutions and corrections. More seriously, as recognized later from the results to be reported, there is a possible inherent fallacy. As growth proceeds the average irradiance per cell decreases with attendant effects upon the photosynthetic characteristics of the cells. Because of the long generation times involved there is a complicating result: cell synthesis in any one time increment is accomplished by cells which have been produced some time earlier under quite different conditions of irradiance. A more reliable method of estimating yields required steady-state conditions. At the same time a new kind of question arose: what cell concentration will give highest yield? The study herein reported arises from this question. In one sense it provides the control experiments for subsequent evaluation of effects of a diffusing cone. In another sense it provides information of more general importance on effects of thickness in a photosynthetic system and reveals characteristics not previously predicted.

Materials and Methods

The growth apparatus used is shown in figure 3. The culture vessel C was a flat-bottomed glass cylinder of 67 mm ID containing 1010 ml of algal suspension at a working depth of 267 mm. It was held at 25° C by immersion in a water bath WB. Mild agitation was provided by aeration with 5 % CO_2 in air via an inlet tube and aided by a magnetic stirrer at the bottom to prevent cell sedimentation. The top of the chamber was fitted with a 50 mm circular diaphragm to define the input light beam and the chamber was enclosed in a nickel-plated sleeve designed to contain the input and exclude ambient illumination.

Light from a 1000-watt projection lamp L and spherical mirror SM was collected by two 6-inch condensing lenses CL. Most of the infra-red was removed by a cooled 15 cm water filter WF containing a 1.9 cm lucite cell of 0.1 M $CuSO_4$. The uniform light field at the back of the condensing lens was focused as a reasonably collimated beam upon the surface S of the growth chamber by a second lens FL and plane mirror M. In order to maintain a reasonably uniform irradiance over the period of a 8 to 12 day experiment the lamp voltage from a ± 0.5 volt regulated supply was adjusted manually from initial value of about 95 to give constant reading of a monitoring photocell P which saw a fraction of the beam diverted by a glass plate G.

At the beginning and end of each experiment the irradiance to the algal chamber was measured in the following way. Irradiance at the position of the input 50 mm diaphragm above S was measured with a large surface Moll thermopile with a compensated Aryton shunt and Rubicon type T galvanometer. It was calibrated without window against a NBS standard lamp but used with a thin glass window and appropriate window correction of 0.91. Estimate of the small amount of radiation above 700 mμ (ca. 3 %) was obtained from readings with and without a Jena RG 8 filter as previously described (11). All values reported describe radiation of less than 700 mμ. Since the 22 mm diaphragm of the thermopile was considerably smaller than the 50 mm light circle to be measured, necessary correction for non-uniformity of the light beam was accomplished by an area comparator made for the purpose. A large area photocell was mounted facing inward at one end of a 3-inch ID, 6-inch long bakelite tube with an inner white paper lining. The other end of the tube held an opal glass diffusing plate under replaceable diaphragms of 22 and 50 mm ID openings. When illuminated by a single lamp 4 meters away the photocell signals for the 2 diaphragms gave a ratio of 5.4 (as compared to an area ratio of 5.1). In practice each thermopile reading was followed by comparator readings and the microwatt input to the pile multiplied by the comparator factor (ca. 4.0) to obtain microwatts over the total 50 mm circle of the light beam. The intent of operation was to hold irradiance in all experiments at about 0.5 cal/cm²—min over the 19.64 cm² input diaphragm area.

The alga used was *Chlorella ellipsoidea* Gerneck (kindly provided by Professor H. Tamiya) and used in preference to *C. pyrenoidosa* because of somewhat cleaner growth characteristics. The medium was a Knop's solution routinely used in this laboratory (11) and known to support growth up to 2 mg/ml without any limitation on specific growth rate. Concentration of algal cells was obtained by centrifuging, washing once in water, and drying at 110° C to constant weight 25 to 50 mg of cells from an appropriate aliquot.

In the first (series I) experiments an approximation to steady-state conditions was obtained by maintaining a fixed regimen of daily harvest and replacement by fresh media. In later (series II) experiments the chamber was chemostatted (9). The subsequent 14 experiments were attended by minor technical difficulties all having to do with prevention of foaming and spattering, maintenance of uniform cell concentration throughout the chamber, and removal of overflow in such a way that chamber and effluent cell concentrations were identical. Finally, the Dow-Corning silicone Antifoam A, up to this point used routinely as an antifoam agent, was abandoned and the following procedure adopted for the last 7 experiments. Uniform increments of about 0.75 ml of fresh media were added at timed intervals of 0.6 to 5.0 minutes. Constant volume was maintained by suction applied for short periods to an overflow device at 3 to 10 minute intervals. The culture effluent was accumulated in a darkened filter flask held at 0° C. Periodic checks on centrifuged cell volumes verified that the concentration of cells in effluent and chamber were identical. An experimental protocol is presented in table I for experiment 48 in which steady-state conditions were reached early in the experiment.

Attending the last 7 chemostatted experiments additional auxiliary measurements were made. After a culture had stabilized at a constant cell concentration small aliquots from the chamber (replaced by effluent) were subjected to chlorophyll assay by the method of Arnon (1). Other small aliquots were used for measurements of photosynthetic characteristics by Warburg manometry. Cells were washed in water and transferred to a Warburg buffer of 0.190 M $KHCO_3$ and 0.010 M Na_2CO_3. Measured rates of O_2 evolution at different values of irradiance were made in the light beam of an optical system essentially like that of figure 3. Spectral character of the light (fig 4) was either identical with that used in the growth apparatus or was limited to a green segment by substitution of 0.5 M $CuSO_4$ and insertion of a Corning 3384 filter. Irradiance was varied by insertion of wire screens and measured by a single juction thermocouple inserted at the position of the Warburg vessel. Measurements of photosynthesis were made in a single vessel during 20-minute exposures to each irradiance, the sequence of irradiances being held in a randomized but constant pattern. Dark respiration was measured simultaneously in duplicate vessels. At the termination of each experiment larger cell samples were prepared in culture medium and respiration measured in conical vessels by the direct method.

At the end of each experiment measurements were made of the transmission at various depths in the growth chamber. An Ektron lead sulfide cell was chosen as a measuring instrument because of relatively flat spectral response (fig 4). It was covered with a 0.25 inch translucent lucite plate and fine wire screen and sealed in a lucite case. Light response of the mounted cell was measured as resistance in a bridge circuit using a 40 volt, 60 cycle input and an AC millivoltmeter. Response was found to be linear and reproducible over a 20-fold range of irradiance. Actual measurements of transmission of the suspension over its 1000-fold range were made by auxiliary use of combinations of neutral metal-film filters of transmissions 0.22, 0.088, 0.040, and 0.040.

TABLE I

PROTOCOL OF EXPERIMENT 48

TIME	HARVEST EFFLUENT	EFFLUENT CONCENTRATION
days	*ml*	*mg/ml*
0.72	329	0.201
1.73	455	0.216
2.76	465	0.226
3.72	422	0.227
4.65	382	0.233
5.64	443	0.234
6.63	440	0.234
7.63	446	0.229
8.70	463	0.230
9.74	453	0.229
10.63	385	0.227
11.66	448	0.238

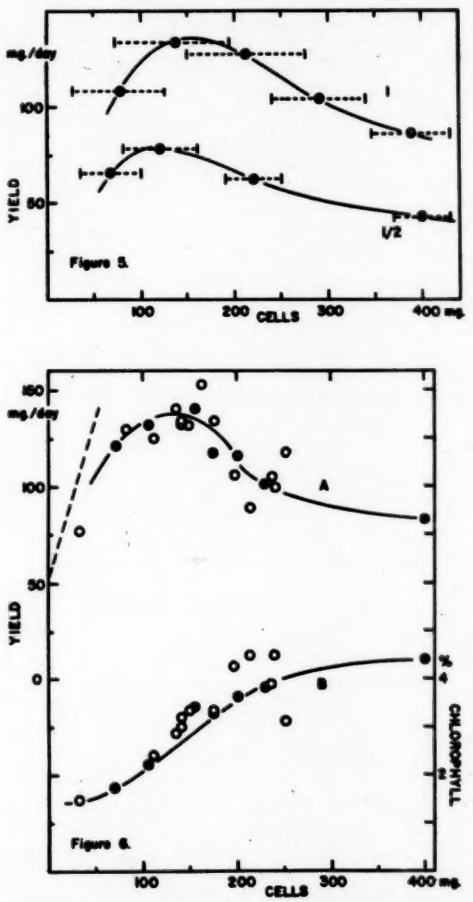
RESULTS

Results of the series I experiments, performed under a regimen of daily dilution and media replacement, are shown in figure 5. The lower curve was determined under one half the standardized irradiance. The upper curve under equivalent full sunlight irradiance showed a relatively narrow optimum occurring at unexpectedly low values of cell concentration. However, because of the large dilution factors required, the resolution was poor and it was reasoned that a closer approach to steady-state conditions would give an even sharper maximum in the curve. Accordingly the refinement of chemostatting was introduced.

The series II chemostatted experiments are presented in figure 6 A. Of the 21 data plotted those of the last 7 experiments are judged more precise and the curve has been drawn with these points strongly weighted. While the precision of measurement is not so high as desired, the data leave no doubt of a relatively sharp maximum in the curve. The auxiliary data presented below and partially summarized in table II provide some understanding of the phenomenon.

Absorption curves for the last 7 experiments are presented in figure 7 as obtained with the Ektron cell. The curves fall short of providing desired information in 2 respects. First the spectral response curve of the Ektron is not perfectly flat though better than that of any other practicable receiver. Secondly, there are shortcomings of the experimental set-up in truly mimicing conditions in a large-area culture exposed to full sunlight. These are due to edge effects. The incident beam fell upon a 19.6 cm² circular area of culture defined by the 50 mm diaphragm. However, scattering by the cell suspension quickly spread the beam over the larger 38.4 cm² area of the chamber cross section. This spreading characteristic accounts for a very rapid initial decrease in irradiance with the result that the curves of figure 7 do not extrapolate to the origin. Furthermore there are unavoidable light losses from the walls of the culture because of imperfect reflection by the surrounding shield.

In spite of the limitations noted above the absorption curves are useful in 2 respects. First, they demonstrate a predictable character of an algal suspen-



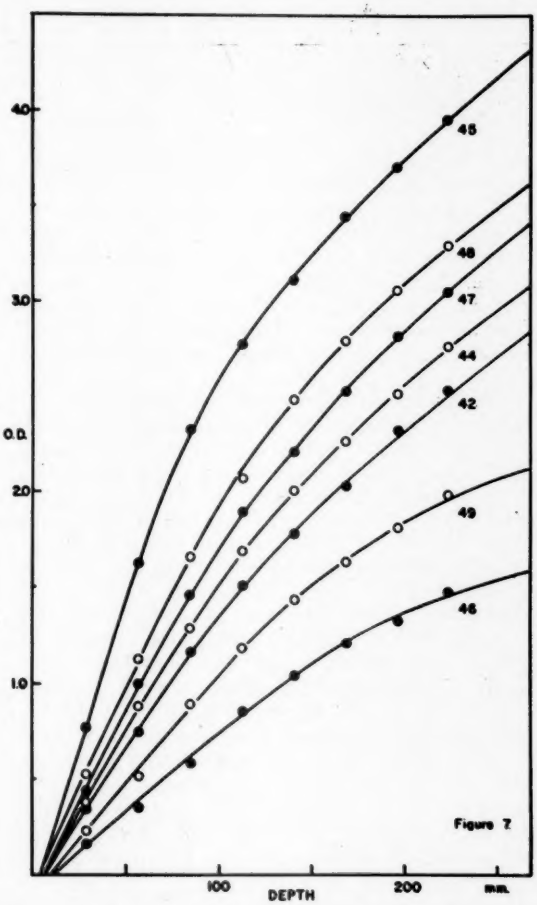


FIG. 5. Yield vs. cell quantity (or cell concentration in mg/1010 ml) for the series I experiments. Each point denotes the constant yield developed in a culture on a fixed daily regimen of partial harvest and media replacement so that cell quantity varied between the limits shown. Lower curve obtained at ½ the equivalent full sunlight irradiance used for the upper curve.

FIG. 6. Yield (A) and chlorophyll content of cells (B) vs. cell quantity (or concentration in mg/1010 ml) for the series II chemostatted experiments. Each point describes steady-state conditions in 1 experiment. Solid points are used for the last 7 experiments which were technically more satisfactory. The dotted line represents a limit on curve A imposed by the maximum specific growth rate.

FIG. 7. Optical density vs. depth for the last 7 series II experiments as obtained with an Ektron cell and calibrated neutral filters.

TABLE II

SUMMARY DATA OF THE LAST SEVEN EXPERIMENTS

EXPT	IRRADIANCE	CELL CONC	YIELD	EFFICIENCY*	SPECIFIC** GROWTH RATE	RATE RESPIRATION	CHLORO-PHYLL CONTENT	Ic***
	kcal/day	*mg/l*	*mg/day*	%	*day*$^{-1}$	*μl O₂/ mg—hr*	%	*μwatts/cm²*
46	15.2	71	126	4.5	1.75	9.6	1.7	8800
49	14.0	104	132	5.1	1.23	7.0	2.2	6000
42	14.4	155	140	5.3	0.90	6.2	3.4	4700
44	14.1	175	117	4.5	0.67	4.2	3.3	4000
47	14.1	202	116	4.5	0.57	4.3	3.6	3600
48	14.1	226	101	3.9	0.43	3.3	3.8	3100
45	14.5	390	87	3.2	0.22	2.1	4.4	2000

* Yield × 0.0054/Irradiance; heat of combustion of cells produced taken as 5.4 cal/mg.
** Yield/1.01 × cell concentration.
*** Average irradiance per cell.

sion. As light passes through the suspension it becomes spectrally limited to a narrow green band for which the cell pigments have a minimum absorption coefficient. Secondly, the curves allow fairly precise estimation of the actual irradiance at each depth in the culture and a function I_C, the average irradiance per cell. Values of I_C cited in table II were obtained from the curves of figure 7 by estimating transmission at each 1 cm layer and taking average transmission X Io.

Irradiance curves of photosynthesis for cells of the last 7 experiments are given in figure 8. Lack of precision reflects known limitations of the procedure employed. However, differences between the curves are so large that 2 characteristics are clearly evident. The curves differ in I_s, however estimated, and in shape. Irradiance curves obtained in green light (fig 4 B) are not plotted since light saturation was not obtained and only 1 useful result was demonstrated. Comparison of the slopes of the curves for green (fig 4 B) vs. "white" (fig 4 A) light allowed estimation that 1.9 μwatt of green light was equivalent to 1.0 μwatt of "white" light in photosynthetic effectiveness. For a white light of the spectral distribution of sunlight the differential would be still greater.

Chlorophyll contents of the series II experiments are plotted in figure 6 B and the data of the last 7 experiments recorded also in table II. Chlorophyll content is an inverse and almost linear function of I_C over the range studied.

DISCUSSION

The essential finding of the present work is the optimum in figure 6 A. The optimum is sharper and occurs at lower cell concentration or higher specific growth rate (table II) than had been expected. Since the ordinate is a first derivative the consequences may be visualized by making an integration to give the same form as the conventional growth curve, drawn as figure 2 B. A curve of shape such as figure 2 A was expected (7). It is of some interest, however, that growth curves similar in shape to figure 2 B have been reported for Chlorella grown under actual sunlight illumination (8).

We attempted to predict the observed yield data of figure 6 by graphical integration of the data of figures 7 and 8 as has been done for natural plankton populations (14). Average irradiance for each of 27 one-cm layers of the suspension were estimated from figure 7 and corresponding values of Q_{O_2} taken from figure 8. Total rate of O_2 production for each experiment was taken as $\Sigma\, Q_{O_2} \times$ mg. Conversion to mg cells/day was made using a factor of 0.78 mg cells/ml O_2 observed in separate balance experiments for *Chlorella pyrenoidosa* growing on nitrate as a nitrogen source. The resulting estimated yield values showed an optimum in the same position as in figure 6 A but with deviations from observed values ranging from zero to +90 %. Introduction of reasonable corrections for green light prevailing in the lower portions of the culture did not materially reduce the deviations. The operation only served to make clear the numerous approximations required. However, a plausible explanation may be framed at a qualitative level.

In figure 6 A the initial rise in the curve is attributable to an increasing fractional absorption of the incident light. The limit established by the broken line is imposed by the light-saturated specific growth rate, taken as 3.0 days $^{-1}$ (16). At high cell concentrations a slow decline in yield is expected since almost all the light is absorbed and further increase in cell concentration can add only to the overhead metabolism or respiration. Both of the above contributions were recognized, at least implicitly, in previous treatments of the problem. A third contribution now is introduced by the present data. Previous treatments (7, 10, 16) have assumed a constant irradiance curve characteristic of the alga used. However, the data of figure 8 make clear that the irradiance curve is not constant; its parameters are functions of cell concentration in the growth chamber. For example in progressing from curve 42 to curve 45 it is clear that the cells described are progressively less effective in using the high irradiance of sunlight at the front surface of a culture.

Of the several possible parameters of the irradiance curves one is chlorophyll content which varies almost linearly with the average irradiance per cell, I_e. Consider a cell moving slowly and at random throughout a culture. Its chlorophyll content is adjusted in response to the average irradiance of the culture. Its photosynthetic response in any position depends upon the irradiance at that position and the irradiance curve (fig 8). A high chlorophyll content, developed in cultures of high cell concentration and low average irradiance, gives rise to a low value of I_s and greater losses due to light saturation. In short, we propose that a third contribution gives rise to the narrow optimum in the yield curve of figure 6 A: the variable character of the irradiance curve controlled in part by chlorophyll content of the cells.

The second part of the argument presented above invokes no principle other than the well known effect of irradiance on chlorophyll content. Indeed the result was anticipated from earlier data of our laboratory (5). However, the argument at this stage is incomplete. In the first place we have no biochemical understanding of the mechanism by which a cell adjusts its chlorophyll content in response to irradiance. And secondly, chlorophyll content as a percentage of dry weight is a crude measure affected by ratio of storage material to photosynethetic machinery. From comparison of shapes of the irradiance curves we must conclude that past history of irradiance of a cell controls some character of the photosynthetic machinery other than its amount.

Direct application of the data to practical conditions of algal growth under actual sunlight illumination is obviously limited. The incident radiation of about 14.3 kcal/day passed through the 19.64 cm² diaphragm is an irradiance of 726 cal/cm²—day or 0.50 cal/cm²—minute and reasonably equivalent to continuous full sunlight. The experiments were designed to study only this limiting case. The maximum yield oberved, 140 mg/day, calculates to 71 g/m²—day if area is taken as the diaphragm area. This estimate is subject to 2 opposing errors. On one hand there are edge effects leading to unmeasured light losses. On the other hand the chamber arrangement, designed to minimize the edge losses, actually introduced an area of culture almost twice as great as the diaphragm area. One might argue that, as viewed by all except the top layer, the effective irradiance should be taken as only about half as great as the measured value. Since we judge the second effect to be more serious than the first, we take 71 g/m²—day as a limit yield achievable by *Chlorella ellipsoidea* at 25° C under continuous full sunlight or a figure about half as great for 12 hour/day full sunlight and not expected to be attained in practice.

We are aware that the principal effect to which this paper is devoted, the variable character of the irradiance curve, is not necessarily a large effect. It introduces variation in yield of less than a factor of 2 even under the limit conditions of our experiments. However, it is a contributing factor too large to be ignored and an important consideration in the search for algae

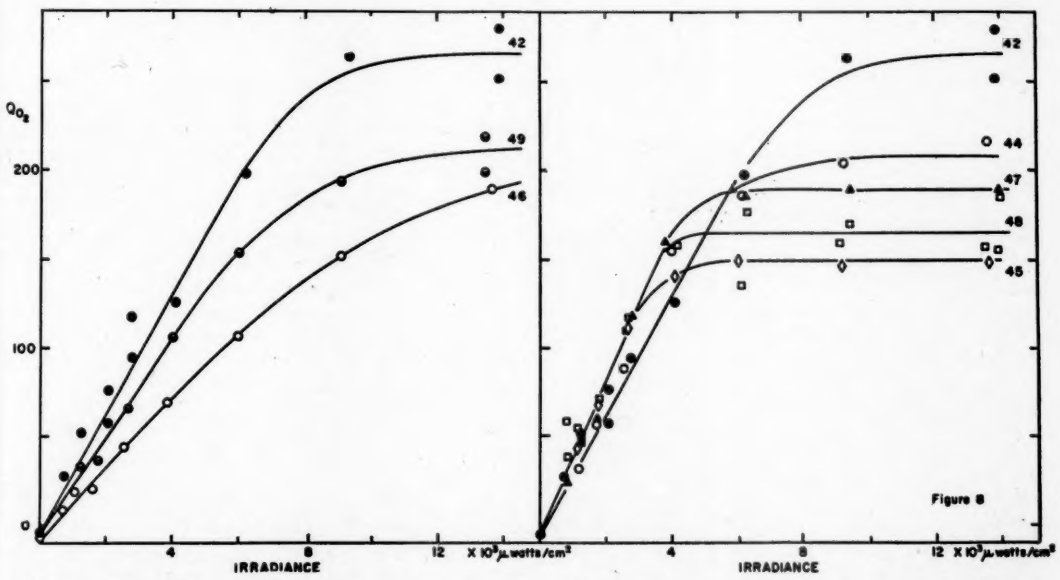


FIG. 8. Rate of photosynthesis, Q_{O_2} in μl O_2/mg—hour, vs. irradiance as measured in Warburg buffer 0.190 M $KHCO_3$ plus 0.010 M Na_2CO_3 for the last 7 experiments. The vessels used contained about 0.5 μl or 0.12 mg of cells per cm² of illuminated area.

which might allow higher yields. For example, it explains why a temperature tolerant alga may be less effective in mass culture under sunlight than originally expected on the basis of an intrinsically higher value of I_s. Sorokin (12) has shown that his Chlorella 71105 has a high value of I_s at 39° C only when grown at a higher irradiance; when grown at a low irradiance such as might be expected as average irradiance in a dense culture, even under incident sunlight, the cells have a much lower value of I_s.

We propose to extend the present study to an examination of the diffusing cone and the use of algae tolerant of higher temperatures.

Summary

An apparatus was devised to allow steady-state growth of *Chlorella ellipsoidea* under continuous visible irradiance equivalent to full sunlight. The culture used was 267 mm deep and 67 mm ID illuminated through a 50 mm circular diaphragm at an irradiance of about 0.5 cal/cm²—min. Yield (mg/day) was observed as a function of cell concentration.

Yield proved to be more sensitive to cell concentration than expected with an optimum of 140 mg/day at about 0.13 mg/ml. Explanation of the optimum was sought from auxiliary measurements of light transmission through the culture, chlorophyll content, and the irradiance curve of photosynthesis. An explanation is proposed in terms of the variable character of the irradiance curve of photosynthesis controlled in part by chlorophyll content of the cells and therefore responsive to average irradiance per cell and cell concentration.

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A SENSITIVE STAGE IN SYNCHRONIZED CULTURES OF CHLORELLA [1]

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Synchronous cultures of microorganisms have been used to study the various stages of development and of physiological activity during the life cycle of the individual cell (cf. 3). Different methods have been proposed to synchronize cultures of Chlorella, e.g. mechanical separation of the cells according to their size (4), pretreatment of the cultures by special light and temperature conditions (4, 13), and regular light-dark changes combined with intermittent dilutions to a standard cell number (5) or with continuous dilution to a standard density (1, 12). The results obtained with cells synchronized in these different ways do not agree in any respect. This may be due to the fact that the applied procedures can influence the physiological activity of the cells apart from synchronizing their life cycle. In a physiological periodicity it may sometimes be difficult to discriminate between exogenous influences and autonomous factors; nevertheless it should be critically tested in any case of synchronization, whether or not an observed effect is definitely bound to the normal development of the cell. This, in our opinion, is particularly desirable with results concerning the chemical composition of the growing cells (e.g. 4), and also with fluctuations of photosynthetic activity occurring in synchronous cultures (1, 10, 12).

As a component in the course of the physiological periodicity of *Chlorella pyrenoidosa* Chick (Emerson strain), observable in synchronous cultures, we found a transient sensitivity to low temperatures. This phenomenon will be described in this paper.

MATERIALS AND METHODS

Cultures of Chlorella were grown in a light thermostat (9000 Lux, 30° C). The glass tubes containing the suspensions were continually flushed from below with a stream of 1.5 vol % CO_2. Complete synchronization has been obtained by a regular change of light and dark periods. With the Emerson strain used and the conditions given, a change of 16 hours illumination and 12 hours darkness (periods of 28 hrs) proved to be better than a 24-hour-periodicity. In this way, the burst of the daughter cells (autospore production) occurring during the dark period 20 to 24 hours after the beginning of each 28-hour-period could always be controlled to its very end. Under the conditions applied the number of daughter cells averaged 10 per mother cell. Four hours of darkness before the next light period proved to be without influence on the further development of the newly formed cells. A long treatment of darkness or insufficient illumination, however, may induce starvation effects and, subsequently, require a rather long recovery time before cell divisions are again possible. At the beginning of illumination the cultures regularly were diluted to a standard cell number (1.56×10^6 per ml, i.e. a rather thin suspension). The course of the life cycle can be seen from figure 3 (solid lines, marked by dots). Continuous dilution or dilution at any other time have proved to be without influence on the position of the cell divisions within the 16/12 hour period.

It may be mentioned that in Chlorella the length of the life cycle is governed mainly by an endogenous (probably inherited) time factor, initiated by the start of illumination. Provided possible starvation effects (e.g. a limiting carbohydrate level) had been excluded, all factors influencing the production of cellular substance (length of the light period, light intensity, temperature, or addition of glucose) did not shift the time of the cell divisions considerably, but strongly affected the number of daughter cells released. This has been described more thoroughly elsewhere (5, 9).

RESULTS

During the period of illumination (16 hrs) synchronized cultures of Chlorella, taken from the light thermostat (30° C), were exposed for 2 hours to a low temperature of 4° C in a parallel thermostat, all other experimental conditions being kept constant. This cold shock proved to have quite different consequences, depending on the stage of development at which the synchronous cells had been treated. At the beginning and at the end of the light period the number of autospores released in one burst was somewhat lower than in the untreated control; 7 to 8 hours after the beginning of illumination (time A, fig 3), however, a cold shock stopped the subsequent cell division completely. Figure 1 shows the number of cells from a series of parallel cultures, treated with 4° C in the intervals mentioned, at the end of the dark period following the cold shock (time B, fig 3); in all cultures the bursts of the cell divisions had definitely ceased at this time. Figure 2 adds a visual impression of this stage, showing portions of the culture tubes in the same sequence as in the columns of figure 1. The pale color of tube IV is not only due to the inhibition of growth but also to a considerable loss of chlorophyll. Microscopic observations at this stage

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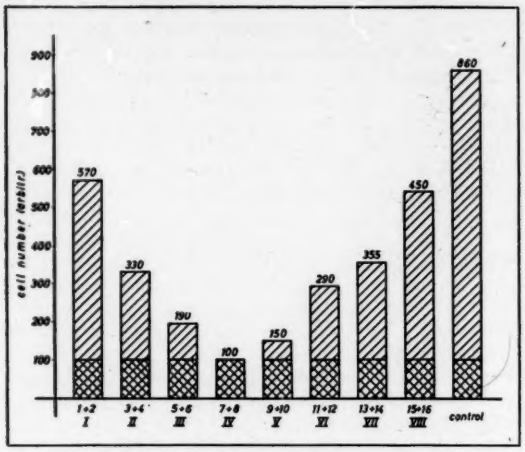

FIG. 1. Number of daughter cells produced in a synchronous culture of Chlorella from a standard number of mother cells (100 = 1.56×10^6 cells per ml) after a 2-hour-treatment with cold at different times of the light period (16 hrs). Cold shock from 30° to 4° C. Cell counts made after the dark period following the light period during which the shocks had been applied (indicated as time A and B respectively in fig 3). The column at the right refers to the untreated control. The most sensitive stage is shown in column IV.

also demonstrated that the cells were partially bleached and relatively poor in living cell matter. This shock effect is shown also in figure 3. Here the untreated control has been compared to the culture shocked during the most sensitive stage (time A). Chlorophyll content, in this case, drops to about 50 %, while in the control it is continually increasing, even during the whole dark period, due to growth of the cells. Figure 3 also shows that during the further course of the experiment growth inhibition and chlorophyll loss are reversible. While the untreated control is continuing its normal life cycle, the shocked sample exhibits at first only a very slight increase in chlorophyll content and a few single cell divisions. During the 2nd light period following the cold treatment, however, chlorophyll develops at a nearly normal rate, while the cell number still remains practically constant. In the subsequent dark period (C in fig 3) cell divisions are also resumed at a fairly normal rate; it appears only that these divisions need a somewhat longer time than they do in the control. In the following periods (not shown in fig 3) all differences had disappeared. It therefore can be stated that complete recovery from cold shock in the sensitive phase had occurred within 75 hours after treatment.

It is remarkable that during the cold treatment in the sensitive stage light seems to be necessary or at least highly conducive to the subsequent manifestation of the described effects. Treated with 4° C at the same stage in the dark (2 hrs), comparable cells only suffer a decrease in the number of autospores as had been found in the case of cultures treated at a less sensitive stage of development (fig 1). It must be mentioned, moreover, that chlorophyll loss is not restricted to the illumination period. In the case of the experiment shown in figure 3, such loss was observed almost exclusively during the dark period. In several other experiments of the same kind the chlorophyll content began to drop even during illumination, but, in any case, this trend did not stop in the dark. We also observed that the reversible loss of chlorophyll sometimes surpassed 50 %. Finally, we found in a few experiments that some of the cold-treated cells died (8), while in the experiment described here (and in many similar ones) dead cells could not be detected. This demonstrates that the reversible inhibition following the treatment occasionally may become lethal, hence irreversible.

CONCLUSIONS

While Chlorella has been generally considered as a fairly stable and resistant organism, synchronous culture reveals that this is not valid during the whole life cycle of the cell. At a relatively early stage of cell development (but not at the very first one) the cells pass through a rather sensitive phase. It remains to be proven by further studies how far this sensitivity is restricted exclusively to a loss of cold resistance. Presumably, other external factors may also be of influence; it does not seem certain whether the cold shock described is primarily due to the low temperature itself, or whether the effect is indirect. In this connection it must be mentioned that light is effective during the induction of the cold shock, whereas the subsequent decrease of chlorophyll content is at least not confined to illumination and thus cannot be explained by a simple photoxidation of the pigment. On the contrary, the disappearance of a part of the chlorophyll may be only a readily apparent symptom of a radical change suddenly induced in the metabolism of the cell. This abnormal metabolism—apparently of a strongly dissimilatory character—does not immediately cause a lethal decay, but remains under cellular control; hence its effects can still be compensated by the normal assimilatory processes which are resumed after some time. It might be interesting to

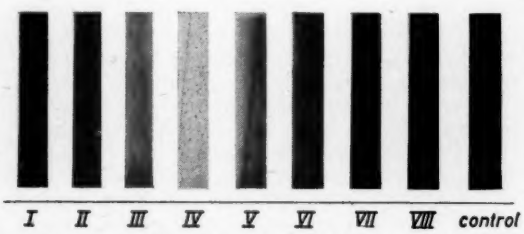

FIG. 2. Portions of the culture tubes after cold shock, photographed at the time where the cell counts (fig 1) were made. Sequence corresponding to the columns of figure 1.

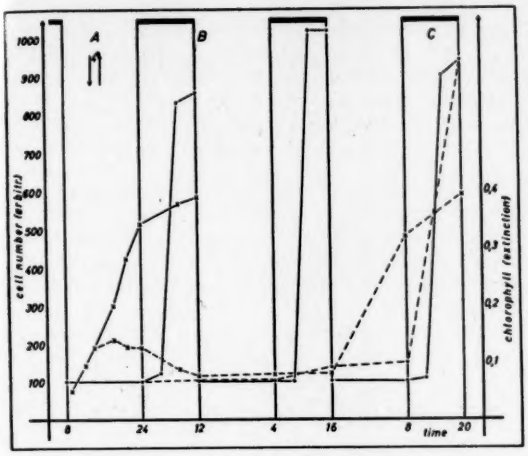

FIG. 3. Cell numbers and chlorophyll content in synchronized cultures of Chlorella, following a cold shock at the most sensitive phase. Solid lines: control. Dashed lines: shocked sample. Dots: cell number. Crosses: chlorophyll. Dark times of the 28-hour-period (16/12) are marked by black bars.

mention that the consequences of the cold shock resemble to some extent certain effects of heat treatment with Euglena (2, 11); but there is a need for more precise studies to corroborate such a similarity.

As to the characterization of the sensitive phase, a cytological effect may be mentioned which has also been found in synchronously grown Chlorella cultures. At a stage almost coinciding with the sensitive phase described here, all nuclei fail to give a positive result with the well-known Feulgen reaction. Conversely, in cultures with continuous illumination only a certain percentage of cells show this negative reaction (6). It may be supposed that transient structural changes of the nuclei, and also of the protoplasm, are responsible for the abnormal behavior in both cases.

Photosynthetic oxygen production, too, is correlated in time with the sensitive phase in some way; during a 16/12 hour light-dark period at 30° C maximal photosynthetic rates have been obtained just at this stage (7). This coincidence, however, cannot prove a causal relationship, since these fluctuations of photosynthetic activity seem to be more directly produced by the external light-dark conditions than by the true life cycle of the cells (7).

Further studies to characterize the sensitive phase and the shock-induced reactions are in progress.

SUMMARY

During the life cycle of *Chlorella pyrenoidosa* (Emerson strain), synchronized by a 16/12 hour light-dark photoperiod, a sensitive stage has been observed after about 7 hours of illumination. Cold shocks (from 30° to 4° C) of 2 hours duration at this stage induce an abnormal cell metabolism, indicated by a transient inhibition of cell growth and cell division, and by a reversible partial loss of chlorophyll.

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EFFICIENCY OF LIGHT ENERGY CONVERSION IN PLANT GROWTH[1]

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It is 20 years ago that Robert Emerson and C. M. Lewis (5) published an outstanding article on the quantum efficiency of photosynthesis. This study showed that Warburg's at that time unsurpassed high value of this efficiency—only 4 quanta required per molecule of CO_2 reduced—could be reproduced, and even improved. At the same time, however, doubt was thrown upon the tacit assumption that the data obtained were representative for true photosynthesis, by showing that the outcome depended strongly on exactly how the data were evaluated.

This paper opened a new era which led to a much wider understanding of the problem, and to a much increased interest in the detailed techniques of the measurements. In the years thereafter, Emerson, on various occasions, has insisted on the importance of the latter point. A general opinion has gradually developed that the efficiency of true, positive, photosynthesis in the majority of cases is not much better than 1 molecule CO_2 for about 10 light quanta absorbed; however, not all the questions which were raised in the various laboratories, and the merits and shortcomings of the various techniques which were employed have yet been elucidated satisfactorily. Kok, from this laboratory, recently has reviewed the field in great detail (17). The above studies represent investigations on the photosynthetic efficiency in its strictest sense. On the other hand there are investigations of a different type but of equal interest, viz., on the efficiency of the outcome of photosynthesis in the long run such as the efficiency of growth, extending from small algal cultures in the laboratory, under controlled conditions, via algal mass cultures, both in the laboratory and in open air, to efficiency of energy conversion in field crops, and ultimately to speculations about the world energy yield on the land and in the oceans.

In our laboratory, we have been working on this type of problem for several years and thus it seems appropriate to survey a few results of the various aspects in this article. The more so, since the great shock of Robert Emerson's sad and unexpected death by itself led us to reflect upon the diverse aspects of the field in which he was deeply interested (6).

The work in our laboratory was initiated after the last world war by calculations of maximal efficiencies for some field crops, taking high annual yields in Holland, from agricultural data, making allowance for roots, stubbles, etc., and comparing them with the average energy on the cultivated surface during the growth period (27). Considering only wave lengths available for photosynthesis, the efficiencies were between 0.5 and 2 %, and the large deviation from photosynthetic efficiency in laboratory experiments of short duration—viz., 20 to 30 %—was the starting point of our work. Earlier computations of efficiency of higher plant growth are discussed in detail in (11). The possible reasons for the relatively low long term efficiency are as follows (27): 1) too high incident light intensities under field conditions; 2) too low CO_2 content of the air; 3) too low temperatures; 4) other limiting factors, e.g., too little water; 5) loss of light between the plants; and 6) factors, provisionally difficult for analysis, e.g., fluctuating rate of photosynthesis, possible "afternoon depression," respiratory losses.

It was stated that several of these factors could be controlled more easily in algal cultures than in the growth of field crops.

EFFICIENCY OF ALGAL GROWTH: The first question to be answered was whether the efficiency of growth, under favorable conditions, could approach closely that of photosynthesis short-term experiments. Work along this line was started in our laboratory in June 1948 by Miss J. T. de Vries, and extended by J. F. Bierhuizen in 1949 using about 0.5 liter Chlorella cultures illuminated by incandescent lights under laboratory conditions, (details in (28)). Over the period of active growth the efficiency of light energy conversion ranged from 12 to 15 % at relatively high light intensities, and from 20 to 24 % at lower light intensities. Thus, it appeared that, under suitable conditions, the efficiency of growth in algal cultures did approach closely that of short-term photosynthesis, being about 25 % (assuming a quantum yield of approximately 0.1). Shortly thereafter, Kok established complete energy balances of algal cultures, cultivated in large Warburg manometer vessels, in sodium light. In Kok's studies all relevant items were measured (12). They confirmed the preliminary data discussed above.

Large scale (300 l) indoor and outdoor mass cultures of algae were set up first in 1950–51 chiefly by van Oorschot. These gave high efficiencies under laboratory conditions, at relatively low light intensities, and high temperatures. A series of 9 experiments gave an average efficiency of 13.3 %, with a maximum of 19.7 %. Outdoor cultures in full daylight showed a much lower yield, viz., 2.6 %, which could be increased to 6.3 % by the application of gauze screens, which reduced the light intensity (19, 28).

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[2] 191st Communication; 70th Comm. on photosynthesis.

A more detailed analysis, using much smaller vessels, showed that under conditions of full summer daylight efficiencies of the order of 8 % were obtained (18). This corresponds to a yield of about 20 g dry weight/m²/day. Similar yields also have been obtained in other laboratories (survey in (24)).

In all our cultures, CO_2-enriched air was applied. After 1954 interest concentrated on several problems that had arisen from previous work: intermittency and high light intensity effects on the photosynthetic apparatus, including spectral changes (13, 14, 15, 16), and the relation between the carbon and nitrogen metabolism of Chlorella (1, 2). With respect to our present discussion, it is of interest that algae growing with a relatively low supply of nitrogen first accumulate carbohydrates, and finally fats, with a continuous decrease in the efficiency of light energy conversion (1, 19).

Recently, we were able to devote renewed attention to the mass culture aspect as such, in relation to efficiency. Previous work had shown that the type of culture vessel was important in obtaining high efficiencies, this effect still is not fully understood. In our recent approach we adopted a technical solution to the stirring problem by introducing washing machines as culture vessels. The type used had a cylindrical transparent lucite container, ("demonstration" model) about 50 cm diameter and 30 cm high (manufacturer: Miele). These provided excellent algal growth. Preliminary series run in the summer of 1958 gave yields of 13 to 22 g dry weight/m²/day, the average efficiencies of the series were from 4.7 to 7.2 %, with daily extremes between 2.0 and 10.5 %. This culture device was very suitable for additional heating, CO_2-supply, and for renewal of part of the suspension. It seems probable that the yields so far obtained may be increased considerably as we learn to improve the composition of the culture solutions and the other conditions. In addition, we are also experimenting with some continuous culture devices which, however, are on a smaller scale. (E. C. Wassink and C. Kaai, to be published).

Efficiency of Higher Plant Growth: Along with this algal work, we have been interested in the efficiency of solar energy conversion in field crops.

Close to the large scale outdoor Chlorella cultures, a grass area of 1 m² in an existing lawn was treated as an experimental plot. Three successive harvests between May and the end of October gave an average efficiency of 2.6 % of the incident, photosynthesizible radiation (28).

Beet seedlings raised in this laboratory by Glas and Gaastra, in 1949, under artificial light of rate-limiting intensities, showed efficiencies of 12 to 19 % (28). More recent experiments under optimal conditions and complete leaf cover of the soil surface gave about the same values; in younger stages the yields were lower (9 to 11 %). Similar experiments in the laboratory under light limiting conditions, using other plants, (turnip and cucumber etc.) showed efficiencies mostly between 7 and 11 %.

Gaastra (7), measured directly the efficiency of photosynthesis of sugar beet leaves under mercury light, with the aid of an infra-red gas analyzer and, by inference, calculated the efficiency in full summer sunlight. The (maximum) efficiency at low light intensities was about 17.8 % (referring to incident light), and about 2 % at full sunlight intensity. At an intensity of 10^4 ergs/cm² sec the rate of photosynthesis was 26 cmm CO_2 per hour per cm² leaf surface which, for high pressure mercury light amounts to a quantum yield of 0.10, confirming earlier observations for leaves which showed maximum values mostly between 0.05 and 0.10 (8, 26).

The annual efficiency of a good beet crop is around 2.2 % (27). Gaastra (7) was the first to point out that this does not necessarily mean that the yield is the same over the entire season. Obviously, the yield cannot be constant, since, in the beginning, the field is scarcely covered while, toward the end, the growth rate slows down considerably. Combining data from growth experiments on sugar beets, performed at Wageningen by Boonstra (3) with corresponding solar energy data (31), Gaastra showed that the efficiency had a maximum of 7 to 9 % in the middle of the season and that between 80 and 90 % of the total organic matter was produced in 45 % of the season (or 2.5 months). This efficiency may be compared with the best values obtained with algae.

Between 1949 and 1951 we carried out a periodic harvest experiment with potatoes in order to establish the trend of the efficiency values. Recently, all the relevant energy data have been worked up. The efficiencies, calculated for surface area actually covered, showed some increase during the 1st half of the season; optimal efficiencies were of the order of 5 %. A more detailed discussion of this experiment is planned elsewhere (30).

In the summer seasons of 1957 and 58, Kamel, in this laboratory, made an elaborate study of growth and efficiency in barley, under natural conditions, employing 4 different light intensities (by artificial shading), and, in separate experiments, 3 different plant densities. The efficiency showed a trend with time similar to the one evaluated for beets by Gaastra (7), being most pronounced at full light intensity. At lower intensities the seasonal efficiency curve was flattened, and the growing period was extended. In the density series, the efficiency curves are all more nearly equal. At first it may seem remarkable that the efficiency of solar energy conversion was highest at the highest light intensity, both in the middle of the season and overall, however, early and late in the season the efficiency declined sharply. The maximum efficiency recorded (on the basis of area actually covered) was about 13.5 % for a period of about 2 weeks in the middle of the season, followed by a sharp decrease. The average efficiency (per cultivated area) over the entire season was 2.9 % for full daylight, and 1.4 % for the lowest light intensity, viz., 25 % of full daylight. The total dry weight per plant in 25 % of full daylight is only about 15 % of that

under full light. Apart from unfavorable morphogenetic reactions at the low light intensity, the integration of slower growth over a long period of time and the, presumed, relatively less positive daily energy balance may tentatively explain these figures. For more details we refer to the original papers (10, 11). Also with sugar beet similar, however, less extensive experiments have been carried out (10, 11).

EFFICIENCY OF PLANT GROWTH ON EARTH AND ITS UTILIZATION BY MAN: Unpublished calculations for efficiency of tree growth in temperate regions on the basis of literature data, carried out by the author showed about the same efficiency values as obtained for field crops, viz. 1 to 2 % of photosynthesizible radiation during the growing season.

Rabinowitch has estimated that per annum about 2.5×10^{23} cal photosynthesizible radiation reaches the earth surface, and that 3×10^{21} cal is fixed by photosynthesis (20, p. 9), so that the overall efficiency is about 1.2 %, including land and oceans. Since 1 ton of carbon is equivalent to about 10^{10} cal, this corresponds to 3×10^{11} tons of carbon. An estimate from certain actual data yields about 1.75×10^{11} tons (20, p. 7). From this figure about 80 % is attributed to the oceans.

For the land area, the estimate is 0.19×10^{11} tons of carbon fixed per annum, which was derived from Schröder's computation (21). This figure may be checked as follows: The wooded area on earth is about 40×10^6 sq km (44×10^6 sq km according to Schröder (21), 36.5×10^6 sq km according to more recent data (25)). Using the data quoted in the 1st paragraph of this section, and assuming that the average production of forests is well reflected by that of temperate regions, the annual yield of a wooded area may be estimated to 10000 kg dry matter per hectare = 10^4 kg per sq km (see also (27)). This would correspond to a carbon yield of 0.4×10^4 kg, or of $36.5 \times 10^6 \times 0.4 \times 10^4 = 1.45 \times 10^{13}$ kg or 0.15×10^{11} tons for the total. The total carbon fixation on the total land area, according to Schröder, is about 1.5 times that of the woods alone, it covers about 150×10^6 sq km. However, about 80×10^6 sq km are areas of low plant production (arid and semi-arid regions). This would, altogether, yield a figure slightly higher than 0.2×10^{11} tons, in very close agreement with Schröder's estimate. Of this total, about one third or 0.07×10^{11} tons might be chiefly agricultural crops, whose cultivated area is about 27×10^6 sq km (21).

The world's human population may be estimated to consume $2.5 \times 10^9 \times 365 \times 2 \times 10^3$ kcal per annum, or 2×10^{15} kcal, in which the average personal daily consumption is set at 2×10^3 kcal.

Stamp (22) has estimated that world's average need for human food is about 10^6 kcal per individual per annum, yielding a slightly higher total figure.

According to our estimations, the annual caloric production of the arable land is about 7×10^{14} kcal, so that the fraction now used for food is about 0.03 or 3 %, and 3×10^{-4} of the solar radiation, assuming a photosynthetic efficiency of 1 %. Thus, the solar radiation in question would amount to 7×10^{18} kcal. In reality, the area outside the woods covers about 60 % of the land area. The land area is about 0.3 of the total earth surface and thus receives about 8×10^{19} kcal of photosynthesizable radiation; 60 % being 5×10^{19} kcal. The average efficiency of photosynthesis outside the woods thus is $7/5 \times 10^{-1} \times 1\% = 0.14\%$. One might say that the "effective cross section" for photosynthesis of the land area outside the woods is 0.14, taking 1 % as a fair average for photosynthesis under natural conditions during the period of growth. Following Schröder's estimation and dividing the land into units of 10^6 sq km, the land area is roughly composed of 40 units of wood, 30 of farmland, 30 of semi-arid land and 50 of arid land. The actual efficiency of the farmland region—assuming that this produces the bulk of the above crop yield—thus would be $110/30 \times 0.14 = 0.5\%$. The same holds, by inference, for the wooded area, and is based on our assumption of 10000 kg per ha average annual production which received support from Schröder's data. Assuming an average growth efficiency of 1 % over large areas, including lost surfaces like rivers, etc. it would mean that the average growing season is 0.5 year which seems reasonable.

We still make the relation: human consumption to photosynthesizable solar energy received. For the total land area, this is $2 \times 10^{15}/8 \times 10^{19} = 0.25 \times 10^{-4}$. It should be mentioned that, according to Stamp (22) only about 30 % of the land area is habitable by man, 20 % being too cold, 20 % too dry, 20 % too mountainous, and 10 % rocky and soilless. According to this estimate, the wood area must be largely outside the potentially habitable area which can be true only in part. Taking Stamp's data, the total human consumption would be equivalent to about 10^{-4} of the solar radiation on this area and probably about twice as high (2×10^{-4}) if wood consumption is included since forests for a considerable part belong to the potentially habitable area.

It is interesting to compare this figure with an estimate for the Netherlands as a country distinguished by a number of interesting peculiarities, viz., 1) the population density has essentially reached a "habitable" upper limit, 2) it has very little area which is in principle unhabitable 3) it is using almost completely any recreation areas and has very few true nature reserves. The area is roughly 3.10^4 sq km, the number of inhabitants 1.1×10^7. Photosynthesizable solar energy per annum is about 3.7×10^{11} kcal per sq km, in total thus 1.1×10^{16} kcal. The amount of food consumed (ca. 3000 kcal/day) is $3.65 \times 10^2 \times 3 \times 10^3 \times 1.1 \times 10^7 = 1.2 \times 10^{13}$ kcal, or 10^{-3} of the photosynthesizible radiation.

If we take the situation in the Netherlands as a rather uncomfortable and nearly impossible situation for Stamp's habitable world area as a whole, this area, on the average, is already at 20 % of this limit (For these considerations it is not very relevant if some food is imported or exported.) According to Daniel's

figures (4) in the U.S.A. the average use of food is only 10^{-5} of incident solar radiation on the entire surface and about 10^{-4} of the radiation on Stamp's inhabitable area, which is near the world average.

The figure about the Netherlands is also interesting because it shows that a dense population has no more food at its disposal than about 0.1 to 0.2 of the potential photosynthetic production on the entire area and, in addition, probably a similar amount of wood. It is easily understandable that this figure is low for several reasons, e.g., 1) areas taken up by roads and social constructions, 2) areas taken by rivers, 3) only part of the crop is food, 4) necessary wood and recreation areas.

This figure for the Netherlands may have an absolute meaning since both agricultural yields per unit area and relative size of arable land are at a maximum. Only food production during the entire year by a more efficient principle (e.g., by very efficient algal cultures) might improve the situation materially. One word may be said about air pollution brought about by a dense population living according to contemporary standards. In the Netherlands, higher plants in general do not yet seem to suffer seriously from air pollution. It may be remarked, however, that lichens, known to be particularly sensitive in this respect, have practically disappeared during the last 30 years.

In the twenties the more conspicuous species, e.g. of the genera Usnea, Ramalina, Evernia and Parmelia, were still also very common in the immediate neighborhood of the smaller cities and villages, whereas now they are not seen any more even in the countryside. There is no scientific proof that this is (only) due to air pollution, but it seems very probable that this is the main reason, and well deserves attention. Apart from the decline in beauty and interest, the loss of the lichens may well mark a first step on a dangerous road, along which other types of organisms also may travel sooner or later.

The number of human beings on earth has increased rapidly during the last century. In the Netherlands there is 1 inhabitant on each 0.3 ha., the world average is 1 on 5 ha. (according to Stamp (22)). It is difficult to say what would be the relation if man still lived as a normal constituent of natural biotypes, like animals do. The primitive civilizations that still exist already have a long human history, and their distance from a truly primitive state is probably larger than that between our present civilization and theirs. However, for our problem, they may serve as a basis for comparison. Thus, e.g., in New Guinea around 1940 1.3×10^6 inhabitants lived on 8×10^7 ha, or 1 on 60 ha, in contrast to Java where it was 1 on 0.3 ha. The New Guinea figure is very low, especially in view of the fact that the greater part of the country may be habitable according to Stamp's considerations. About the same figure holds at present for Canada (22), which, may be for different reasons, however, as a large part of the country may be too cold for permanent habitation.

The figure for the U.S.A. is about the world average (22), which is roughly 10 times larger than the New Guinea figure, and about 10 times smaller than the figure for the Netherlands. It should be observed that the U.S.A. has a large inhabitable area. It is interesting that the increase in population up to this level in the U.S.A. has already entailed the loss of most of the virgin aspects of nature (c.f. e.g., the disappearance of the prairie type of vegetation, and also of the virgin forests (9)).

The above considerations primarily dealt with a consideration of plant growth on land. We will now consider briefly the oceans. Schröder had estimated the production of the ocean to be about the same total as that of the land (the oceans are roughly 3.5×10^8 sq km against 1.5×10^8 sq km for the land; the yield per unit area thus would be about 3/7 that of the land). Rabinowitch (20) quotes a much higher figure, viz., about 1.6×10^{11} tons against 0.2×10^{11} tons for the land area. This figure was mainly based upon Riley's data; it corresponds to about 0.9 % efficiency of solar energy conversion for the entire ocean area during the whole year.

More recently, Steemann Nielsen (23) arrived again at a much lower figure, viz. 55 g C/m² per annum. This would amount to a total of 0.2×10^{11} tons, almost exactly as Schröder had estimated; it would bring down the average efficiency of solar energy conversion to about 0.11 % for the whole year for the entire ocean area. The average efficiency for the entire land area is about 0.27 %, according to our preceding computations, for the whole year.

It would seem more satisfactory that the yield of the ocean per unit surface area comes out less than that of the land since, on the average, the ocean is definitely less green than the land. This is not compensated by the thick layer of water in which organism may live, for looking from the surface, it would have to give a densely green (or brown) impression in order to give a high yield.

We can estimate, therefore, that the world production of plant material is around $2 \times 0.2 \times 10^{11}$ or 0.4×10^{11} ton C per annum, equivalent to 0.4×10^{18} kcal, or about 1/7 of Rabinowitch's estimate, viz. 3×10^{18} kcal. The average efficiency of solar energy conversion on the entire earth surface thus would amount to 1/7 of the Rabinowitch figure, viz. $1/7 \times 1.2 = \sim 0.17$ %.

The resources man derives from the sea are estimated between 0.5 and 1 kg/ha per annum. This is excessively low as compared with the land which yields roughly 2 ton C per sq km or 50 kg dry CH_2O per ha. The 0.5 to 1 kg for the ocean may correspond to ~0.1 kg dry weight, or ~500 times less than the profit of land vegetation.

I have previously tried to estimate the entire production of the sea from average fish catches (28), assuming that 1 kg of fish is derived ultimately from 25 kg of phytoplankton, that 1 % of all fish is caught, and that 1 % of the living matter in the ocean is fish. This yields an annual production of 2.5×10^4 kg dry matter or 10^4 kg C per ha per annum, or 10^8 kg C per

sq km. For the entire area of the ocean this would be $3.5 \times 10^8 \times 10^6 = 3.5 \times 10^{14}$ kg C per annum, or 3.5×10^{11} ton. This would double the Rabinowitch figure for the oceans, which seems very improbable. However the assumptions employed were only approximations. Perhaps much more than 1 % of all fish are caught, or possibly much more than 1 % of all living matter in the ocean is fish. It would be worth while to assemble data with respect to these assumptions which would help us to substantiate further the recent estimates on photosynthesis in the oceans, and thus on the total yield of plant growth on earth.

The discussion in the last section may serve as a preliminary approach to the problems outlined. A full evaluation will require a thorough comparison and consideration of detailed data on the various fields of production, and on climatic influences, which is beyond the scope of this article. Moreover, detailed analyses should first be extended in various directions. It is hoped, however, that the order of magnitude of the computed values is correct.

Summary

Algal growth, in principle, may constitute a very efficient source of solar energy conversion. Also in large cultures, efficiencies of 7 to 10 % with regard to the photosynthesizible part of full intensity solar radiation have been obtained.

Higher plant growth, in principle, is no less efficient, but during part of the season while inefficient periods are necessarily included. The overall yield during a season mostly lies between 1 and 2 %.

Weakening of the light intensity may result in an increase in efficiency, other conditions permitting.

Estimations of yield and efficiency of solar energy conversion by plant growth on the entire earth have been discussed, as well as some relations with its use by man. The most probable figure for the yield of plant growth on earth appears to be around 0.4×10^{11} tons of carbon per annum, half of which is produced on the land, and half in the oceans. The average efficiency of conversion of photosynthesizable radiation is around 0.17 %, that of the land area $\wedge$ 0.27 %, and that of the ocean $\wedge$ 0.11 %. Density of human population is unlikely to be able to increase beyond utilization for nutrition of $\wedge$ 0.1 % of photosynthesizable radiation on the habitable land area.

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News and Notes

Canadian Society of Plant Physiologists: At a meeting in Saskatoon, Saskatchewan on October 27, 1958, the Canadian Society of Plant Physiologists was founded. The new society grew out of a series of Annual Research Conferences on Plant Physiology that had been held at various Canadian universities and research institutions over a period of eight years. The officers of the new society are: Paul R. Gorham, President; E. R. Waygood, Vice-president; D. Siminovitch, Secretary-Treasurer; R. O. Bibbey, Eastern Director; Stewart A. Brown, Western Director. Correspondence should be addressed to The Secretary-Treasurer, Chemistry Division, Canada Department of Agriculture, Ottawa, Canada.—David Siminovitch, *Vice-president.*

Gordon Research Conference: The Third Gordon Research Conference on Biochemistry and Agriculture will be held at Kimball Union Academy in Meridan, N. H. on August 3–7, 1959. Conference co-chairmen, R. S. Bandurski and G. L. McNew, have put together a program which extends over a wide range of topics. Sessions have been arranged on the following principal topics; the biochemical basis for ecological responses, the role of phosphorus in plant nutrition, metabolism of adenyl anhydrides. nucleic acid metabolism, polysaccharide synthesis, chemical inhibition of metabolism, influence of ionizing radiation on biochemical processes, and the regulation of plant growth. Among the conference participants will be several distinguished scientists from Canada and from abroad. Since admission to the conference is by invitation, interested persons should write for application forms and information from; Dr. W. G. Parks, Chemistry Dept., University of Rhode Island, Kingston, R. I.

Sociedad Argentina de Fisiologia Vegetal: The Sociedad Argentina de Fisiologia Vegetal was formed on December 13, 1958, at a meeting held at the Faculty of Agronomy, University of La Plata. The following officers were elected: President, Enrique Sivori, University of La Plata; Vice-president, Jorge Morello, University of Tucuman; Secretary-treasurer, Miguel Raggio, Institute for Scientific and Technological Research; Councillors, Alberto Soriano, University of Buenos Aires and Clara Phoebe Rumi, University of La Plata.—G. R. Noggle, *Executive Secretary-treasurer.*

Plant Physiology: The first 4 volumes of PLANT PHYSIOLOGY have just been reprinted by the Johnson Reprint Corporation. Since these volumes have been out of print for some time, their current availability will be welcomed by a number of institutions that lack them in their collections. For additional information contact: Johnson Reprint Corporation, 111 Fifth Avenue, New York 3, New York.—G. R. Noggle, *Executive Secretary-treasurer.*

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The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Correspondence concerning membership and its privileges, dues, change-of-address, etc., should be addressed to the Executive Secretary-Treasurer, Dr. G. R. Noggle, ASPP, Box 2665, University Station, Gainesville, Florida. Business correspondence regarding subscriptions, back numbers, change-of-address of subscribers, etc., may be addressed to Plant Physiology, Box 749, Lancaster, Pa., or to the Executive Secretary-Treasurer at the address above. All matters pertaining to nominations, elections, programs, and meetings of the society should be addressed to the Secretary, Dr. Walter D. Bonner, Jr., Cornell University, Ithaca, New York. Correspondence with reference to editorial matters should be addressed to the Editor, Dr. Allan H. Brown, Department of Botany, University of Minnesota, Minneapolis 14, Minnesota.